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(71) Applicant: **THE JAPANESE RESEARCH AND DEVELOPMENT, ASSOCIATION FOR THE IMPROVEMENT OF, ENZYME FUNCTION IN FOOD INDUSTRY**
4F Minesawa Building, 17-17 Kodenma-cho, Nihonbashi
Chuo-ku, Tokyo 103(JP)

(72) Inventor: **Murakami, Kohji, c/o Chuo Kenkyusho**
The Green Cross Corporation, 1180-1, Shodai
Otani-2-chome, Hirakata-shi(JP)
Inventor: **Ishida, Yutaka, c/o Chuo Kenkyusho**
The Green Cross Corporation, 1180-1, Shodai
Otani-2-chome, Hirakata-shi(JP)
Inventor: **Masaki, Atsushi, c/o Chuo Kenkyusho**
The Green Cross Corporation, 1180-1, Shodai
Otani-2-chome, Hirakata-shi(JP)
Inventor: **Kawabe, Haruhide, c/o Chuo Kenkyusho**
The Green Cross Corporation, 1180-1, Shodai
Otani-2-chome, Hirakata-shi(JP)
Inventor: **Arimura, Hirofumi, c/o Chuo Kenkyusho**
The Green Cross Corporation, 1180-1, Shodai
Otani-2-chome, Hirakata-shi(JP)
Inventor: **Tatsumi, Hiroki**
c/o Kenkyuhonbu of Kikkoman Corporation
399, Noda, Noda-shi(JP)
Inventor: **Murakami, Seiji**
c/o Kenkyuhonbu of Kikkoman Corporation
399, Noda, Noda-shi(JP)
Inventor: **Nakano, Eiichi**
c/o Kenkyuhonbu of Kikkoman Corporation
399, Noda, Noda-shi(JP)
Inventor: **Motai, Hiroshi**
c/o Kenkyuhonbu of Kikkoman Corporation
399, Noda, Noda-shi(JP)

(74) Representative: **Coleiro, Raymond et al**
MEWBURN ELLIS 2 Cursitor Street
London EC4A 1BQ(GB)

(54) Gene expression unit comprising the promoter and the terminator as well as the gene of alkaline protease.

(57) A novel alkaline protease promoter and alkaline protease terminator which each effectively function in a host-vector system of molds can be obtained from yellow koji mold. A gene expression unit comprising the promoter and terminator is used to construct an expression vector capable of expressing an useful substance in molds such as Aspergillus oryzae, Aspergillus niger, Aspergillus awamori, Aspergillus sojae, etc.

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containing 3 intron sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a restriction enzyme map of genome gene of alkaline protease from yellow mold.
Fig. 2 shows a nucleotide sequence of genome gene of yellow mold alkaline protease containing yellow mold alkaline protease promoter at the 5′-end.
Fig. 3 shows a nucleotide sequence of genome gene of yellow mold alkaline protease containing yellow mold alkaline protease terminator at the 3′-end.
Fig. 4 shows the entire nucleotide sequence of genome gene of yellow mold alkaline protease.
Fig. 5 shows procedures for ligating alkaline protease genome genes.
Fig. 6 shows procedures for constructing expression vector pAP045.

DETAILED DESCRIPTION OF THE INVENTION

Hereafter the present invention is described in detail.

The genome gene of alkaline protease and the promoter and terminator in accordance with the present invention are prepared as follows.

Chromosomal DNA is extracted from yellow mold (Aspergillus oryzae) by known methods (for example, Oakley et al., Gene, 61, 385-399 (1987)). The extracted chromosomal DNA is partially digested with a suitable restriction enzyme. After introduction of the digested gene into a plasmid vector (e.g., pUC19, etc.), host (e.g., Escherichia coli strain JM109, HB101, etc.; manufactured by Takara Shuzo Co., ltd.) is transformed with the vector to obtain a genomic library. By screening using a probe (e.g., cDNA probe, a synthesis probe, etc.), positive clone is obtained and the desired plasmid DNA is recovered. After digesting the plasmid DNA with a suitable restriction enzyme, the digestion product is introduced into a plasmid vector (e.g., pUC19, etc.) to transform therewith a host (e.g., Escherichia coli strain JM109 HB101). Thus, the desired clone is obtained.

As a plasmid in which a gene fragment containing the promoter of the present invention has been incorporated, there may be pAP017 (of. restriction enzyme map in Fig. 6). As a plasmid in which a gene fragment containing the terminator of the present invention has been incorporated, there may be pAP025 (cf. restriction enzyme map in Fig. 6).

The nucleotide sequence of DNA was analyzed by known methods (for example, the Maxam-Gilbert method, the dideoxy method).

The thus obtained genome gene of alkaline protease from yellow mold according to the present invention, structure of which was determined as described above, has a restriction enzyme map shown in Fig. 1 and a nucleotide sequence shown in Fig. 4. In addition to the promoter and terminator domains of the present invention, the genome gene also has 3 intron sequences, which are shown by IVS 1 through 3 in the figures.

The promoter of the present invention is present at the 5′ site of the genome gene and also has 1110 bp DNAs. The nucleotide sequence is shown in Fig. 2 and corresponds to nucleotide No. 1 (C) to 1110 (C) in the nucleotide sequence of genome gene shown in Fig. 4.

In addition to DNA sequence shown in Fig. 2, the present invention also includes other DNA sequences having the function equivalent to that of the promoter, such as DNA sequence having a nucleotide sequence partly different from the nucleotide sequence shown in Fig. 2, DNA sequence containing a part of the nucleotide sequence shown in Fig. 2 and DNA sequence containing at least the nucleotide sequence described above.

The terminator of the present invention is present at the 3′ site of the genome gene and has 530 bp DNAs. The nucelotide sequence is shown in Fig. 3 and corresponds to nucleotide No. 2458 (G) to 2988 (G) in the nucleotide sequence of genome gene shown in Fig. 4.

In addition to DNA shown in Fig. 3, the present invention also includes other DNA sequences having the function equivalent to that of the terminator, such as DNA sequence having a nucleotide sequence partly different from the nucelotide sequence shown in Fig. 3, DNA sequence containing a part of the nucleotide sequence shown in Fig. 3 and DNA sequence containing at least the nucleotide sequence described above and.

By ligating the promoter or terminator with a suitable plasmid vector, the promoter or terminator may be utilized as an expression vector in yellow mold.

These promoter and terminator of the present invention may be used in one set as a gene expression

non-translation region at the 3'-end. These oligonucleotices were synthesized with a DNA synthesizer (381A) (manufactured by Applied Biosystems Co., ltd.), using the reagents and method indicated by the manufacturer. Radioactive label of the synthetic oligonucelotides was made using [γ-^{32}P]ATP (manufactured by Amersham Co., Ltd.) and T4 polynucelotide kinase (manufactured by Takara Shuzo Co., Ltd.).

3. Southern hydridization

Using the synthetic oligonucleotide probe prepared in the preceding 2. above, the chromosomal DNA of Aspergillus oryzae ATCC 20386 was analyzed for alkaline protease gene, in accordance with the Southern hybridization method. Firstly, the chromosomal DNA of Aspergillus oryzae was digested with several restriction enzymes (for example, BamH I, Eco RI, etc.). Thereafter, isolation was performed by agarose gel electrophoresis. After the electrophoresis, DNA was subjected to blotting onto a nitrocellulose filter by the Southern transfer method. The Southern transfer method was performed according to the method of Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). Hybridization was performed at 42 °C in 6 x SSC (0.9 M NaCl, 0.09 M trisodium citrate), 0.5% sodium lauryl sulfate, 5 x Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.01 M EDTA (pH 8.0), and a solution of 100 µg/ml transfer RNA. The probe was added in a concentration of about 1.0 x 10^7 cpm/ml. Washing was carried out at 45 °C with 6 x SSC and 0.5% sodium lauryl sulfate solution 3 times. After air-drying the filter, the filter was applied to an X-ray film (manufactured by Fuji Photo Film Co., Ltd., RX) at -80 °C to obtain autoradiogram. As the result, a single and clear band was obtained respectively with both probes of AP-23 and AP-24, for the digestion products of Aspergillus oryzae chromosome with restriction enzymes.

4. Preparation of library

As the result of Southern hybridization described above, a band of about 6.5 kb was noted with the digestion product of Aspergillus oryzae-derived chromosomal DNA with Bgl II in the case of using AP-23 as the probe and a band of about 4.5 kb was noted with the digestion product with Hind III in the case of using AP-24. Bgl II and Hind III are restriction enzymes present in alkaline protease cDNA. It is thus considered that cloning of DNA fragments obtained with both restriction enzymes would cover cloning of alkaline protease gene over almost the entire region. Therefore, genomic library of Aspergillus oryzae using these restriction enzymes was prepared. Firstly, about 200 µg of the chromosomal DNA of Aspergillus oryzae prepared in 1, above was digested at 37 °C with 500 units each of Bgl II and Hind III (both manufactured by Takara Shuzo Co., Ltd.) overnight. After 0.8% agarose gel electrophoresis (at 30 V overnight), DNA fragment having a size of 5.0 to 7.0 kb (kilobase) and DNA fragment having a size of 3.0 to 5.0 kb were extracted and purified from the Bgl II digestion product and the Hind III digestion product, respectively, based on the size of a molecular weight marker simultaneously subjected to electrophoresis. Extraction and purification from agarose gel can be performed by the method of Maniatis et al. (supra). On the other hand, after digestion with BamH I which formed the same cohesive end as with Bgl II or digestion with Hind III using pUC19 as the vector, the terminus was dephosphorylated with alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd.) to prevent self ligation.

The DNA fragment of 5.0 to 7.0 kb as the Bgl II digestion product described above was mixed with pUC19 previously digested with BamH I, and the DNA fragment of 3.0 to 5.0 kb as the Hind III digestion product was likewise mixed with pUC19 previously digested with Hind III. Using "Ligation Kit"(manufactured by Takara Shuzo Co., Ltd.), ligation was performed. Competent cells (manufactured by Takara Shuzo Co., Ltd.) of Escherichia coli strain HB101 were transformed using the resulting mixture. A part of the transformed Escherichia coli was subjected to plating on L agar medium (1% Bacto-trypton, 0.5% yeast extract, 1% NaCl, 1.5% agar) supplemented with 50 µg/ml of ampicilin for determining frequency of transformation. The balance was supplemented with 50-fold volume of L-broth (1% Trypton, 0.5% yeast extract, 1% NaCl) supplemented with 50 µg/ml of ampicilin and cultured at 37 °C overnight to amplify the colony. The colony was then stored at -80 °C as genomic library of Aspergillus oryzae.

5. Colony hybridization

Colony hybridization was performed as described below. Firstly, the genomic library of Aspergillus

bromophenol blue) was added to the reaction mixture to terminate the reaction.

Then, 1 to 3 µl of the reaction solution obtained above was applied onto 6% acrylamide-urea gel. At the same time, the solution obtained by the dideoxy reaction using pAP017 as a template and AP-26 as a primer was applied to the same gel as a size marker. As the result, it is revealed that 3 bands were noted with the reaction subjected to the primer extension and 3 transcription initiation sites were present in the alkaline protease gene. The results are shown in Fig. 2. The sequence TATAAAT which is considered to be so-called TATA box is present at the upstream by 30 to 40 nucelotides from the uppermost stream of the transcription initiation site thus determined. At the upstream by 80 to 90 nucelotides, the sequence CCAAAT which is considered to be CAAT box is present.

8. Ligation of alkaline protease genome gene

To ligate the alkaline protease genome genes cloned to the two DNA fragments (pAP017 and pAP025), the procedures shown in Fig. 5 were performed. Firstly, about 50 µg of pAP017 was digested with Nco I (manufactured by Takara Shuzo Co., Ltd.) and the terminus was rendered blunt, using "DNA Blunting Kit" (manufactured by Takara Shuzo Co., Ltd.). With the digested fragment was mixed 5 µg of BamH I linker (manufactured by Takara Shuzo Co., Ltd.). Using "DNA Ligation Kit" (manufactured by Takara Shuzo Co., Ltd.), the both were ligated with each other. After purification by ethanol precipitation, DNA was digested with BamH I and Hind III (manufactured by Takara Shuzo Co., Ltd.). The DNA mixture was isolated by 1% agarose gel electrophoresis. The DNA fragment of about 1200 bp containing the 5′-site of alkaline protease genome gene was recovered and purified from the gel. Recovery of DNA from the gel can be performed according to the method of Maniatis et al. (supra). On the other hand, after digesting about 50 µg of pAP025 with Pst I (manufactured by Takara Shuzo Co., Ltd.), the terminus was rendered blunt, using "DNA Blunting Kit" (manufactured by Takara Shuzo Co., Ltd.). Likewise, 5 µg of BamH I linker was mixed with the digested fragment. Using "DNA Ligation Kit" (manufactured by Takara Shuzo Co., Ltd.), ligation was performed. After the ligation, purification was carried out by ethanol precipitation and DNA was digested with BamH I and Hind III.

The DNA digestion product was isolated by 1% agarose gel electrophoresis. The DNA fragment of about 1800 bp containing the 3′-site of alkaline protease genome gene was recovered and purified from the gel.

About 3 µg of the aforesaid 5′-site DNA fragment (about 1200 bp, BamH I/Hind III fragment) and about 3 µg of the 3′-site DNA fragment (about 1800 bp, BamH I/Hind III fragment) of alkaline protease were mixed with each other. After ligation by "DNA Ligation Kit", digestion was performed with BamH I. The digested DNA was isolated by 1% agarose gel electrophoresis and the DNA fragment having the desired size (about 3 kb) was recovered and purified. After the DNA fragment containing this alkaline protease genome gene was mixed with the BamH I-digested pUC19 to ligate them, the ligation product was transferred to competent cells of Escherichia coli strain JM109 (manufactured by Takara Shuzo Co., ltd.). A transformant which acquired ampicillin resistance was screened to obtain clone bearing the desired plasmid pAP1725. pAP1725 is DNA inserted with alkaline protease genome gene (about 3 kb) at the BamH I site of pUC19.

9. Preparation of expression vector

Expression vector using the promoter and terminator of alkaline protease gene was prepared by strategy shown in Fig. 6.

For isolating the terminator domain, 10 µg of pAP025 was digested with Afl II (manufactured by Takara Shuzo Co., Ltd.). Thereafter, the cleaved site was converted into the blunt end, using "DNA Blunting Kit" (manufactured by Takara Shuzo Co., Ltd.). After purification by ethanol precipitation, further digestion was performed with Pst I (manufactured by Takara Shuzo Co., Ltd.). The digested fragments were isolated by 1% agarose gel electrophoresis. Among them, DNA fragment (about 500 bp) in the terminator domain of alkaline protease was extracted and purified from the agarose gel. On the other hand, after pUC19 which may be used as a vector was digested with Pst I and Hinc II (manufactured by Takara Shuzo Co., Ltd.), the cleaved site was dephosphorylated with alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd.). The cleaved vector (about 100 ng) was mixed with the terminator DNA fragment (about 500 bp) described above. After ligation of the mixture by "DNA Ligation Kit", Escherichia coli obtain JM109 (manufactured by Takara Shuzo Co., Ltd.) was transformed with the mixture. From the clone which acquired ampicillin resistance, plasmid DNA was extracted and screened to obtain the desired plasmid pAP044.

40
CCATGGTTAT TCTGCGGAAG CGAAACCACC CTCCCACCCA

80
AACAGGGCTA ATGTGCCCAG GTCCTGATAC CATCAGAAGA

120
CCTCCAGGAG CACATGCCTG TTCGCATAAC CGTGGTGTAG

160
CACCAGGAAT TGCTTAGCTT AGCTTCTTCG ACTGGGGGGC

200
CAGAAAGTGC TTATCGCAAA GATCCCATCC CTTTGTGTGA

240
TAGCCCCTCC CGCGGCCCTT GATCAAGCCG TTCTCGCTCG

280
CCCATACCGA AACCGCGATA TTATAGGTGC ACATGGTTAT

320
TATTCTTTTT CTTTTTCTTT TTCTTTGCTT CTCATGCAGC

360
CCCATACGTT GCCGAATTTG GCTACACCTT GGGGCTCATT

400
CTTCGAAGTT TAGATTCCGA CAAGACCTCA GCACCCAATC

440
AAAACCCTTG ATTCCTGATA AAAGACGTGG AAAAAAGCGG

480
ATATCGCGTG AGGATGCCAA GCAAAGGGAA TGGGTCACAT

520
TGATCTCTGT CGCGCTGTTA GGATGATCTT CACTCCTAAA

GATTAGATTC CGGAAAATGA ATTAGGGCTG GCGTTCCAAC
TCCTGGGGAG TGCCGATGTT ACTGTACTTT ATGAAAGAAA
GTAAGTCTAT TGGTACACAG CTGCAG

5. A gene expression unit comprising an alkaline protease promoter of Claim 1 or 2 and an alkaline protease terminator of claim 3 or 4.

6. A genome gene of alkaline protease from yellow mold, containing domains of an alkaline protease promoter of claim 1 and an alkaline protease terminator of claim 3 and containing 3 intron sequences.

7. A genome gene of alkaline protease according to claim 6, which is represented by the restriction enzyme map in Fig. 1.

8. A genome gene of alkaline protease according to claim 6 or 7, which has the following nucleotide sequence.

760
CTTGCCATAG CCTTGTGTTC TTCACGGTCT ATCGGAACAC

800
CCGTTCATGA CTGAAAGGGG TCAGCGTCCG TGGTGGTCAA

840
CATCATTCTC ATCTTTCATC ATGCCCGCTG ATTGATAGAG

880
TAATTTCCGG TGGAGCACAA CGCCGTCCTC TGAGATGCAA

920
TGTCACCCTG TAAGTTTCAA CTACAATCTG TAGTACAGAG

960
CATCCTTGTC ATTGCATGCT GTGCAAGTGA TCCAAATCCG

1000
TAGAACTTGC TCGAGAACAG GGAAATATAG AACTCCTGAA

1040
GGTTATAAAT ACCACATGCA TCCCTCGTCC ATCCTCACTT

1080
CCATCATCAA GCCAGCGGTT TCTATCCTCC GACTTGAGTT

1120
GTTCTTGCGC ATCTTTACAA TCTTCTCATC ATGCAGTCCA

1160
TCAAGCGTAC CTTGCTCCTC CTCGGAGCTA TCCTTCCCGC

1200
GGTCCTCGGT GCCCCTGTGC AGGAAACCCG CCGGGCCGCT

1240
GAGAAGCTTC CTGGAAAGTA CATTGTCACA TTCAAGCCCG

1280
GCATTGACGA GGCAAAGATT CAGGAGCATA CCACCTGGGC

1320
TACCAACATT CACCAGCGCA GTCTGGAGCG TCGTGGCGCC

1360
ACTGGCGGTG ATCTTCCTGT CGGTATTGAG CGCAACTACA

1400
AGATCAACAA GTTCGCCGCC TATGCAGGCT CTTTCGACGA

1440
TGCTACCATT GAGGAGATTC GCAAGAACGA AGATgtttgt

2200
TCTGCCCCTG ATGCCATCAC TGTTGCCGCT ATCCAGAAGA

2240
GCAACAACCG CGCCAGTTTC TCCAACTTTG GCAAGGTCGT

2280
TGACGTCTTC GCTCCCGGTC AAGATATCCT TTCTGCCTGG

2320
ATTGGCTCTT CCTCTGCCAC CAACACCATC TCTGGTACCT

2360
CCATGGCTAC TCCCCACATT GTCGGCCTGT CCCTCTACCT

2400
CGCTGCCCTT GAGAACCTCG ATGGCCCCGC TGCCGTGACC

2440
AAGCGCATCA AGGAGTTGGC CACCAAGGAC GTCGTCAAGG

2480
ATGTTAAGGG CAGCCCTAAC CTGCTTGCCT ACAACGGTAA

2520
CGCTTAAGTA CCAGGAGTAC GTCGCAGGAT TCTACCATTG

2560
TTACTGGAAT ACAATGATGA TTAGAAAACG AAGAGCGTTA

2600
TGATTCGGAC GGATATATGC ATGGCACCCA TACAGCGTGA

2640
TACATAGGCT GTTTGCTCAA GAATTAGGAT TTTATCTGAA

2680
TCCATGTACA GAGTATACTT ATGTTAGTAG TCAATAAAAT

2720
CTTGGCTTTC TAATTTTGTC CGATCTACAA GGGGTCGTCG

2760
ATCACAGAAC GAACTAGATG TGCAGGGGAC GATGATCACC

2800
CGTTCTTAGC AAGACCTCTA GTAGTTGTCG ACCATAGCTT

2840
TGACGCGAAT CATGACCCTA CTACTTCTAG ATTGCAGACC

2880
AAGTCGCATG ACAATGTCCT CTTTGGATTA GGATTAGTAG

FIG. 1

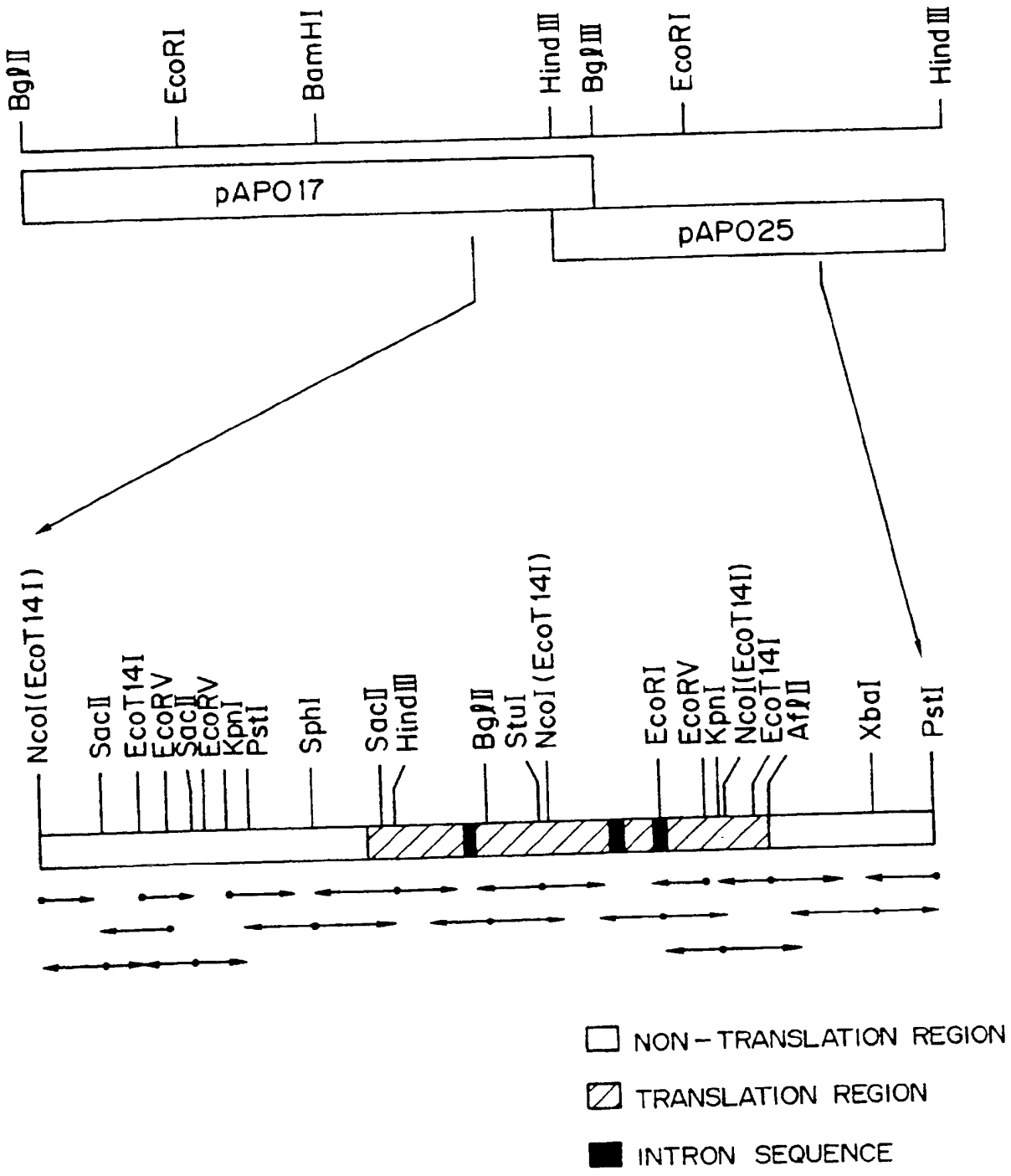


FIG. 3

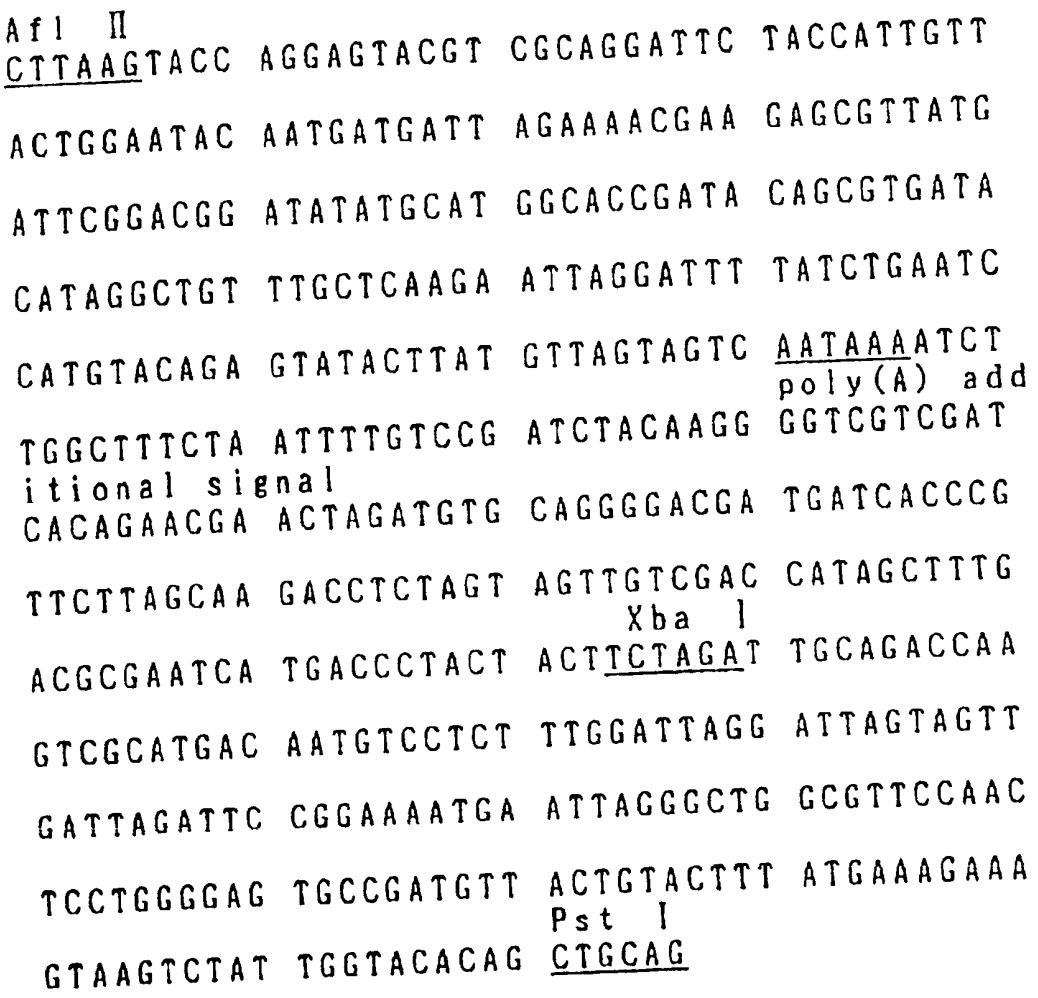
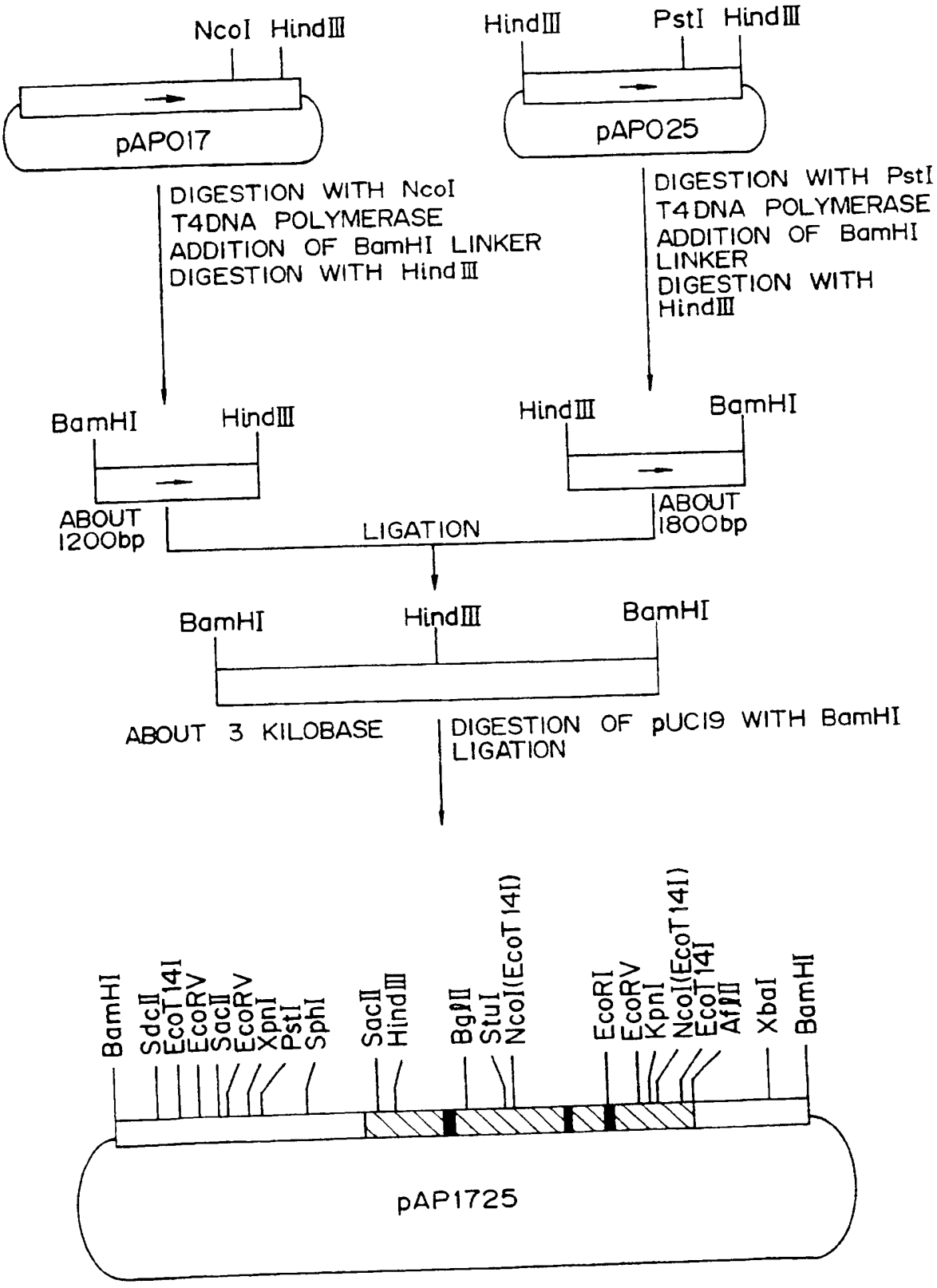


FIG. 4B


1270 1280 1290 1300 1310 1320 1330 1340 1350
CAGGAGCATACCACCTGGGCTACCAACATTCACCAGCGCAGTCTGGAGCGTCGTGGCGCCACTGGCGGTGATCTTCCTGTCGGTATTGAG
GlnGluHisThrThrTrpAlaThrAsnIleHisGlnArgSerLeuGluArgArgGlyAlaThrGlyGlyAspLeuProValGlyIleGlu
1360 1370 1380 1390 1400 1410 1420 1430 1440
CGCAACTACAAGATCAACAAGTTCGCCGCCTATGCAGGCTCTTTCGACGATGCTACCATTGAGGAGATTCGCAAGAACGAAGATgtttgt
ArgAsnTyrLysIleAsnLysPheAlaAlaTyrAlaGlySerPheAspAspAlaThrIleGluGluIleArgLysAsnGluAsp
1450 1460 1470 1480 1490 1500 1510 1520 1530
ggtcatccgctcgcatttttgaatgacagctaactcgcgcccagGTTGCCTACGTCGAGGAGGACCAGATCTACTACCTCGATGGCCTGA
I V S 1
ValAlaTyrValGluGluAspGlnIleTyrTyrLeuAspGlyLeuT
1540 1550 1560 1570 1580 1590 1600 1610 1620
CTACCCAGAAGAGTGCCCCCTGGGGTCTGGGCAGCATTTCCCACAAGGGCCAGCAGAGCACCGACTACATCTACGACACTAGTGCCGGCG
hrThrGlnLysSerAlaProTrpGlyLeuGlySerIleSerHisLysGlyGlnGlnSerThrAspTyrIleTyrAspThrSerAlaGlyG
1630 1640 1650 1660 1670 1680 1690 1700 1710
AGGGCACCTATGCCTACGTGGTGGATAGCGGTGTCAATGTCGACCATGAGGAGTTCGAGGGCCGCGCCAGCAAGGCCTACAACGCTGCCG
luGlyThrTyrAlaTyrValValAspSerGlyValAsnValAspHisGluGluPheGluGlyArgAlaSerLysAlaTyrAsnAlaAlaG
1720 1730 1740 1750 1760 1770 1780 1790 1800
GTGGTCAGCATGTGGACAGCATTGGCCATGGCACCCACGTTTCCGGCACCATTGCTGGCAAGACTTATGGTATCGCCAAGAAGGCCAGCA
lyGlyGlnHisValAspSerIleGlyHisGlyThrHisValSerGlyThrIleAlaGlyLysThrTyrGlyIleAlaLysLysAlaSerI
1810 1820 1830 1840 1850 1860 1870 1880 1890
TCCTTTCGGTCAAAGTTTTCCAGGGTGAATCGAGCAGCACTTCCGTCATTCTTGACGGCTTCAACTGGGCTGCCAACGACATTGTTAGCA
leLeuSerValLysValPheGlnGlyGluSerSerSerThrSerValIleLeuAspGlyPheAsnTrpAlaAlaAsnAspIleValSerL
1900 1910 1920 1930 1940 1950 1960 1970 1980
AGAAGCGTACCAGCAAGGCTGCAATCAACATGAGCTTGGgtgagtttacattgttcttctctacttggaacgcgcgagcgctaatttcaa
ysLysArgThrSerLysAlaAlaIleAsnMetSerLeuG
I V S 2
1990 2000 2010 2020 2030 2040 2050 2060 2070
aaacacagGCGGTGGCTACTCTAAGGCTTTCAACGATGCGGTCGAGAACGCATTCGAGCAGGGTGTTCTCTCGGTTGTCGCTGCCGGTAA
lyGlyGlyTyrSerLysAlaPheAsnAspAlaValGluAsnAlaPheGluGlnGlyValLeuSerValValAlaAlaGlyAs

FIG. 5



European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 30 9821

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	AGRIC. BIOL. CHEM., vol. 52, no. 7, 1988, pages 1887-1888; H. TATSUMI et al.: "Cloning and sequencing of the alkaline protease cDNA from Aspergillus oryzae" * Whole article *		C 12 N 15/57 C 12 N 15/80
P,A	MOLECULAR AND GENERAL GENETICS, vol. 219, no. 1-2, October 1989, pages 33-38; H. TATSUMI et al.: "A full lenght cDNA clone for the alkaline protease from Aspergillus oryzae: Structural analysis and expression in Saccharomyces cerevisiae" * Whole article *		
A	EP-A-0 238 023 (NOVO INDUSTRI A/S) * Page 4, lines 1-7; page 5, lines 18-19; page 6, lines 1-8; claims *		

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 12 N

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	12 December 90	HUBER A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
P : intermediate document
T : theory or principle underlying the invention

E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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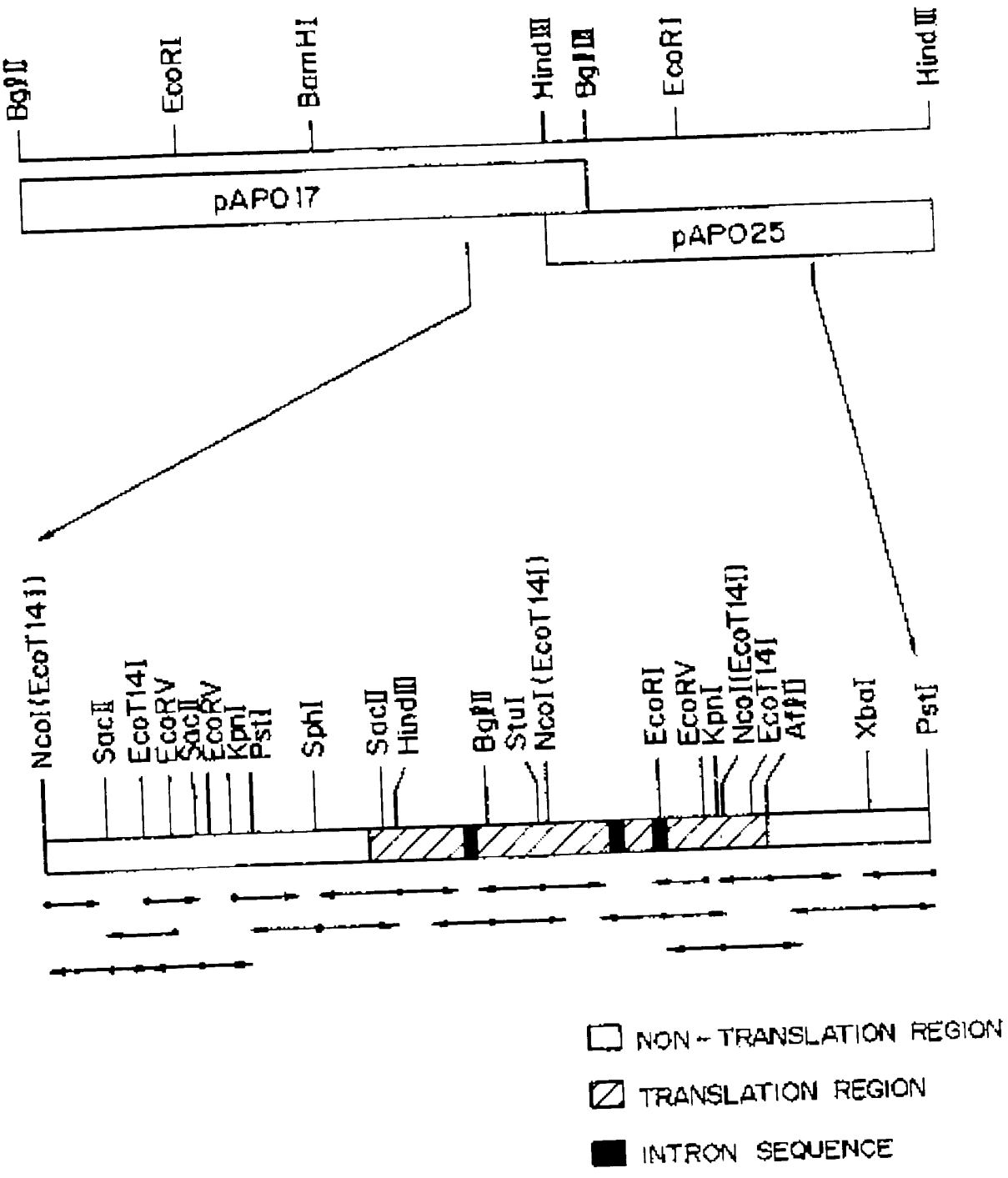
& : member of the same patent family, corresponding document

FIG. 1


BglII
EcoRI
BamHI
HindIII
BglII
EcoRI
HindIII
pAPO17
pAPO25
NcoI(EcoT14I)
SacII
EcoT14I
EcoRV
SacII
EcoRV
KpnI
PstI
SphI
SacII
HindIII
BglII
StuI
NcoI(EcoT14I)
EcoRI
EcoRV
KpnI
NcoI(EcoT14I)
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AflII
XbaI
PstI
NON-TRANSLATION REGION
TRANSLATION REGION
INTRON SEQUENCE

FIG. 3

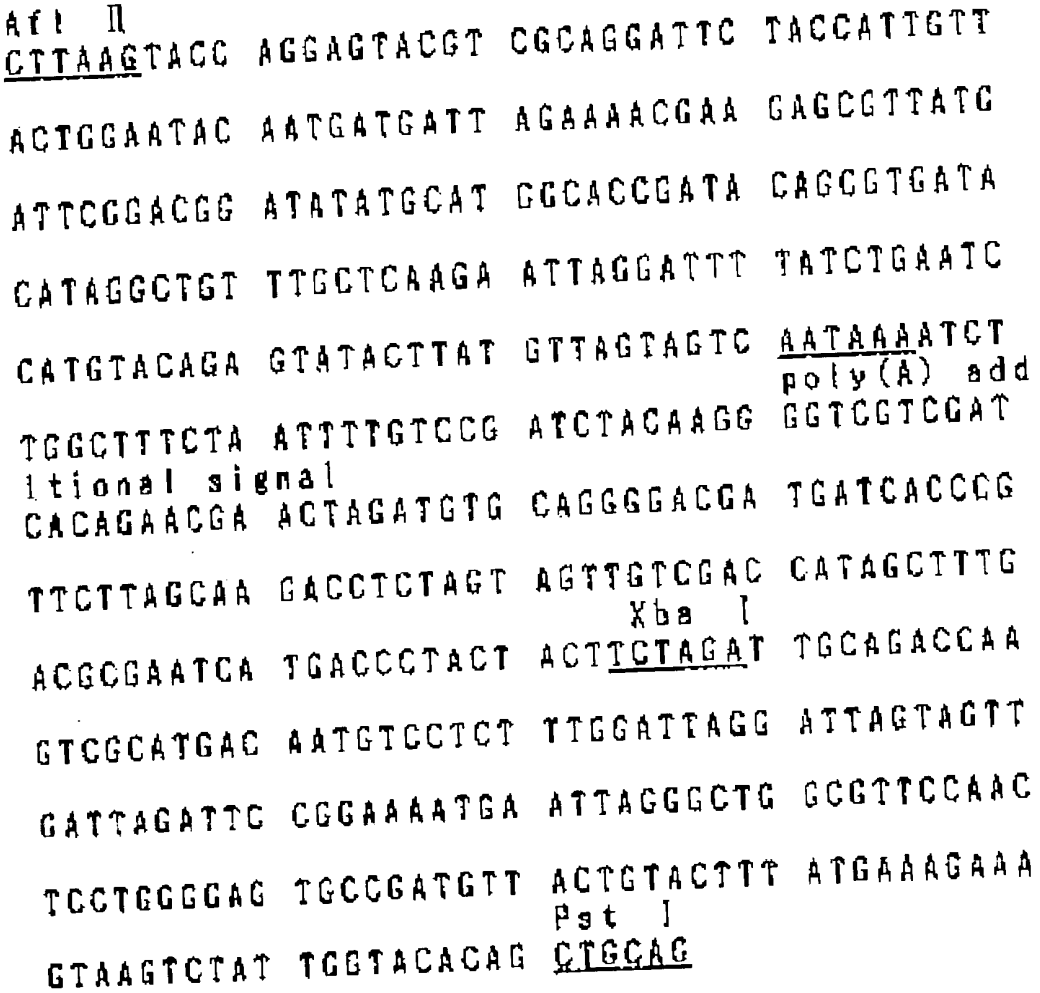

FIG. 4B

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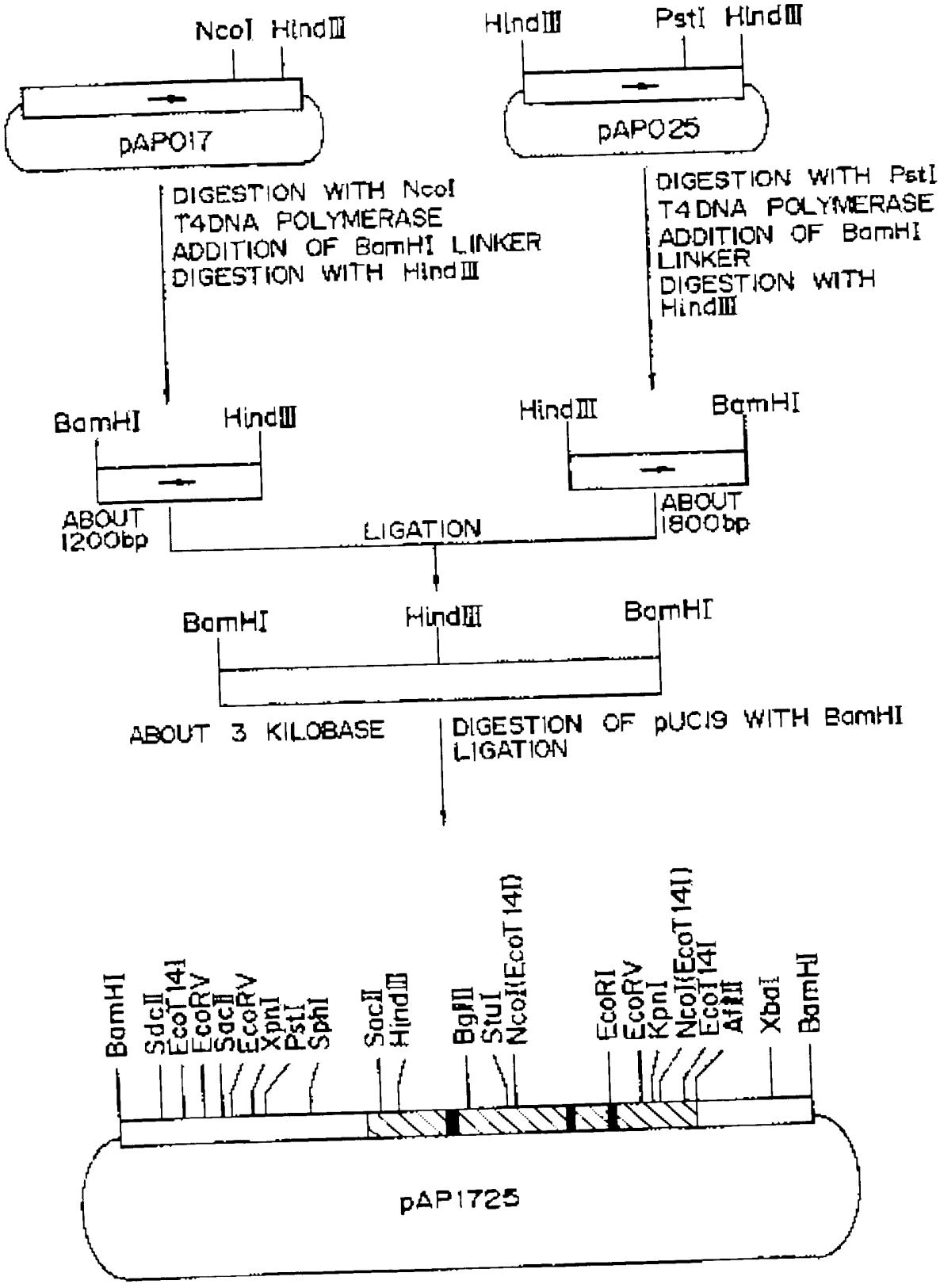
FIG. 5



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(54) Gene expression unit comprising the promoter and the terminator as well as the gene of alkaline protease

Aus Promoter, Terminator und dem Gen der alkalischen Protease bestehende Genexpressionseinheit

Unité d'expression de gène comprenant le promoteur, le terminateur et le gène de la protéase alcaline

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(73) Proprietors:
- **THE GREEN CROSS CORPORATION**
  Osaka-shi Osaka (JP)
- **KIKKOMAN CORPORATION**
  Noda-shi (JP)

(72) Inventors:
- **Murakami, Kohji, c/o Chuo Kenkyusho**
  Otani-2-chome, Hirakata-shi (JP)
- **Ishida, Yutaka, c/o Chuo Kenkyusho**
  Otani-2-chome, Hirakata-shi (JP)
- **Masaki, Atsushi, c/o Chuo Kenkyusho**
  Otani-2-chome, Hirakata-shi (JP)
- **Kawabe, Haruhide, c/o Chuo Kenkyusho**
  Otani-2-chome, Hirakata-shi (JP)
- **Arimura, Hirofumi, c/o Chuo Kenkyusho**
  Otani-2-chome, Hirakata-shi (JP)
- **Tatsumi, Hiroki**
  399, Noda, Noda-shi (JP)
- **Murakami, Seiji**
  399, Noda, Noda-shi (JP)
- **Nakano, Eiichi**
  399, Noda, Noda-shi (JP)
- **Motai, Hiroshi**
  399, Noda, Noda-shi (JP)

(74) Representative: **Coleiro, Raymond et al**
MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP (GB)

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Description

The present invention relates to a novel alkaline protease promoter and an alkaline protease terminator derived from a yellow mold, especially yellow koji mold, a gene expression unit comprising the promoter and the terminator, and relates to a genome gene of alkaline protease.

The structure of alkaline protease gene derived from Aspergillus oryzae which is one of the yellow molds is unknown. To our knowledge, the gene itself has not yet been isolated.

Alkaline protease is a hydrolase which acts on protein or its partial hydrolysate to decompose the peptide bond and, has been widely used as a drug, and in refreshments, detergents, etc.

As a result of various investigations on Aspergillus oryzae-derived alkaline protease gene, the present inventors succeeded for the first time in isolating Aspergillus oryzae-derived alkaline protease gene and prepro type alkaline protease gene and determining their structures, based on which patent applications were filed (Japanese Patent Application Nos. 63-51777 and 63-170018). However, these genes are those derived from mRNA of alkaline protease. Any such publication disclosing the genome gene of alkaline protease and its production does not disclose its structure.

On the other hand, Escherichia coli, Bacillus subtilis, yeast, animal cells, etc. have been heretofore studied as hosts capable of producing substances using genetic engineering. However, each of these hosts involves advantages and disadvantages. In particular, in the case of producing protein containing a sugar chain or protein having a certain steric structure, Escherichia coli, Bacillus subtilis and yeast are not suitable as hosts. In animal cells, costs for cultivation are high and many problems are involved in practical production. Thus, attention has recently been brought to an expression system using a mold as host. For example, according to Upshall et al., Bio Technology, 5, 1301-1304 (1987), TPA (tissue-type plasminogen activator) which was not expressed in Escherichia coli or yeast in its active form is expressed in mold (Aspergillus nidulans) in its active form.

Under such circumstances, it has become significant to develop a promoter or terminator effectively functioning in the host-vector system of molds.

An object of the present invention is to obtain a genome gene of alkaline protease from yellow mold, analyze its structure to determine promoter and terminator domains of the genome gene and newly obtain from the genome gene a promoter and a terminator effectively functioning in the host-vector system of molds and provide a gene expression unit comprising the promoter and the terminator.

As a result of extensive investigations, the present inventors have newly obtained the genome gene of alkaline protease, analyzed its structure and succeeded in identifying the promoter domain present at the 5'-end of the genome gene and the terminator domain present at the 3'-end of the genome gene. The present invention has thus been accomplished.

A first aspect of the present invention provides a plasmid comprising a promoter sequence derived from the *Aspergillus oryzae* alkaline protease genome gene, which sequence is as shown in Figure 2, or a functional equivalent thereof.

A second aspect of the present invention provides a plasmid comprising a terminator sequence derived from the *Aspergillus oryzae* alkaline protease genome gene, which sequence is as shown in Figure 3, or a functional equivalent thereof.

A third aspect of the present invention provides a gene expression unit comprising the alkaline protease promoter and the alkaline protease terminator.

A fourth aspect of the present provides a plasmid comprising a genome gene of alkaline protease derived from *Aspergillus oryzae*, the gene having the restriction enzyme map shown in Figure 1.

A fifth aspect of the present invention provides a plasmid comprising a genome gene of alkaline protease derived from *Aspergillus oryzae* and having the nucleotide sequence shown in Figure 4.

The invention thus enables determination of the structure of a genome gene of alkaline protease from yellow mold, which gene contains domains of the alkaline protease promoter and the alkaline protease terminator and containing 3 intron sequences.

In the accompanying drawings:

Fig. 1 shows a restriction enzyme map of genome gene of alkaline protease from yellow mold.

Fig. 2 shows a nucleotide sequence of genome gene of yellow mold alkaline protease containing yellow mold alkaline protease promoter at the 5'-end.

Fig. 3 shows a nucleotide sequence of genome gene of yellow mold alkaline protease containing yellow mold alkaline protease terminator at the 3'-end.

Fig. 4 shows the entire nucleotide sequence of genome gene of yellow mold alkaline protease.

Fig. 5 shows procedures for ligating alkaline protease genome genes.

Fig. 6 shows procedures for constructing expression vector pAP045.

Hereinafter embodiments of the present invention are described in detail.

The genome gene of alkaline protease and the promoter and terminator in accordance with the present invention

are prepared as follows.

Chromosomal DNA is extracted from yellow mold (Aspergillus oryzae) by known methods (for example, Oakley et al., Gene, 61, 385-399 (1987)). The extracted chromosomal DNA is partially digested with a suitable restriction enzyme. After introduction of the digested gene into a plasmid vector (e.g., pUC19, etc.), host (e.g., Escherichia coli strain JM109, HB101, etc.; manufactured by Takara Shuzo Co., ltd.) is transformed with the vector to obtain a genomic library. By screening using a probe (e.g., cDNA probe, a synthesis probe, etc.), positive clone is obtained and the desired plasmid DNA is recovered. After digesting the plasmid DNA with a suitable restriction enzyme, the digestion product is introduced into a plasmid vector (e.g., pUC19, etc.) to transform therewith a host (e.g., Escherichia coli strain JM109 HB101). Thus, the desired clone is obtained.

A plasmid in which a gene fragment containing the promoter of the present invention has been incorporated is pAP017 (cf. restriction enzyme map in Fig. 6). A plasmid in which a gene fragment containing the terminator of the present invention has been incorporated is pAP025 (cf. restriction enzyme map in Fig. 6).

The nucleotide sequence of DNA was analyzed by known methods (for example, the Maxam-Gilbert method or the dideoxy method).

The thus obtained genome gene of alkaline protease from yellow mold, the structure of which was determined as described above, has a restriction enzyme map shown in Fig. 1 and a nucleotide sequence shown in Fig. 4. In addition to the promoter and terminator domains utilised in the present invention, the genome gene also has 3 intron sequences, which are shown by IVS 1 to 3 in the figures.

The promoter of the present invention is present at the 5' site of the genome gene and also has 1110 bp DNAs. The nucleotide sequence is shown in Fig. 2 and corresponds to nucleotide No. 1 (C) to 1110 (C) in the nucleotide sequence of genome gene shown in Fig. 4.

In addition to DNA sequence shown in Fig. 2, plasmids of the present invention may also include other DNA sequences having the function equivalent to that of the promoter, such as DNA sequence having a nucleotide sequence partly different from the nucleotide sequence shown in Fig. 2, DNA sequence containing a part of the nucleotide sequence shown in Fig. 2 and DNA sequence containing at least the nucleotide sequence described above.

The terminator present in a plasmid of the present invention is present at the 3' site of the genome gene and has 530 bp DNAs. The nucleotide sequence is shown in Fig. 3 and corresponds to nucleotide No. 2458 (G) to 2988 (G) in the nucleotide sequence of genome gene shown in Fig. 4.

In addition to DNA shown in Fig. 3, plasmids of the present invention may also include other DNA sequences having the function equivalent to that of the terminator, such as DNA sequence having a nucleotide sequence partly different from the nucleotide sequence shown in Fig. 3, DNA sequence containing a part of the nucleotide sequence shown in Fig. 3 and DNA sequence containing at least the nucleotide sequence described above.

By ligating the promoter or terminator with a suitable plasmid vector, the promoter or terminator may be utilized as an expression vector in yellow mold.

These promoter and terminator utilised in the present invention may be used in one set as a gene expression unit.

By introducing a gene coding for urokinase, hepatitis B antigen, human serum albumin, interferon α or interferon γ or a gene coding for its derivative into the resulting expression vector, further transforming therewith hosts such as Aspergillus oryzae, Aspergillus niger, Aspergillus awamori, Aspergilus sojae, etc. and culturing the transformants, the desired compounds can be obtained.

According to the present invention, the alkaline protease promoter and terminator, and the gene expression unit comprising the promoter and terminator as well as the genome gene of alkaline protease can be newly provided. In particular, the promoter and terminator, and the gene expression unit comprising the promoter and terminator can be used to construct an expression vector useful for molds such as Aspergillus oryzae, Aspergillus niger, Aspergillus awamori, Aspergillus sojae, etc., by ligating with a suitable plasmid vector. The present invention thus greatly contributes to production of useful substances using the host-vector system of molds.

Hereafter embodiments of the present invention are described in more detail with reference to the Examples below.

Examples

1. Preparation of chromosomal DNA from Aspergillus oryzae

To extract chromosomal DNA from Aspergillus oryzae, the following procedures were carried out. Firstly, one platinum ear loop of Aspergillus oryzae strain ATCC 20386 was taken from slant and inoculated on 50 ml of YPS medium (1% yeast extract, 2% Bactopeptone and 2% soluble starch) followed by incubation at 30°C for 2 days. The culture broth was further inoculated on 500 ml of YPS medium charged in an Erlenmeyer's flask of 3 liter volume. Incubation was carried out at 30°C for one day. The hyphae were collected by filtering through 3 laminated sheets of gauze. The collected cells were washed twice with 20 mM EDTA solution (pH 8.0). After washing further once with extraction buffer (100 mM Tris-hydrochloride, pH 8.0, 10 mM EDTA, 1% sodium lauryl sulfate), water was removed through filter paper.

The wet cells were mixed with the same weight of sterilized sea sand B (manufactured by Nacalai tesque Co., Ltd.). The mixture was homogenized in a mortar for about 5 minutes. To the homogenate was added 60 ml of the extraction buffer (supra) and, the mixture was again homogenized for further 5 minutes. The homogenized cells were centrifuged in a centrifuging tube. The supernatant was transferred to a new centrifuging tube. RNase A (manufactured by Sigma Co.) was added to the supernatant in a final concentration of 5 μg/ml to react at 60°C for 20 minutes. 1/6-Fold volume of 3 M potassium acetate solution was added to the reaction mixture. After allowing to stand at room temperature, centrifugation was performed and the supernatant was transferred to a new, sterilized centrifuging tube. The supernatant was extracted twice with the same volume of TE-saturated phenol (equilibrated with the same volume of a solution containing 100 mM Tris-hydrochloride and 10 mM EDTA, pH 8.0) and then 3 times with the same volume of ether. To the solution after the extraction, 2-fold volume of ethanol was slowly added so as not to trouble the interface and the chromosomal DNA formed at the interface was wound around a Pasteur pipette. The wound DNA was rinsed, in sequence, with 70% ethanol, 90% ethanol and 100% ethanol. After drying, the DNA was dissolved in TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 8.0).

2. Synthesis of probe

Generally in the case of cloning genomic gene using cDNA, hydridization is often performed using cDNA itself as a probe. However, where cDNA is used as a probe under a situation that the desired gene forms a family or has a pseudogene, its specificity becomes low. Therefore, for cloning of alkaline protease gene, oligonucleotide synthesized based on the non-translation region of the cDNA sequence (Japanese Patent Application No. 63-170018) at the 5'- and 3'-ends was used as a probe. The sequence of synthesized oligonucleotide is shown below.

AP-23: 5' GCG CAA GAA CAA CTC AAG TCG GAG GAT AGA 3'

AP-24: 5' CAT GTA CAG AGT ATA CTT ATG GTA GTA GTC 3'

AP-23 is an oligonucleotide having a size of 30 nucleotides (30-mer) which was designed based on the sequence of alkaline protease cDNA (Japanese Patent Application No. 63-17018) in the non-translation region at the 5'-end. AP-24 is a 30-mer oligonucleotide which was designed based on the sequence in the non-translation region at the 3'-end. These oligonucleotides were synthesized with a DNA synthesizer (381A) (manufactured by Applied Biosystems Co., ltd.), using the reagents and method indicated by the manufacturer. Radioactive label of the synthetic oligonucleotides was made using [γ-^{32}P]ATP (manufactured by Amersham Co., Ltd.) and T4 polynucleotide kinase (manufactured by Takara Shuzo Co., Ltd.).

3. Southern hydridization

Using the synthetic oligonucleotide probe prepared in procedure 2. above, the chromosomal DNA of Aspergillus oryzae ATCC 20386 was analyzed for alkaline protease gene, in accordance with the Southern hybridization method. Firstly, the chromosomal DNA of Aspergillus oryzae was digested with several restriction enzymes (for example, BamH I, Eco RI, etc.). Thereafter, isolation was performed by agarose gel electrophoresis. After the electrophoresis, DNA was subjected to blotting onto a nitrocellulose filter by the Southern transfer method. The Southern transfer method was performed according to the method of Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). Hybridization was performed at 42°C in 6 x SSC (0.9 NaCl, 0.09 M trisodium citrate), 0.5% sodium lauryl sulfate, 5 x Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.01 M EDTA (pH 8.0), and a solution of 100 μg/ml transfer RNA. The probe was added in a concentration of about 1.0 x 10^7 cpm/ml. Washing was carried out at 45°C with 6 x SSC and 0.5% sodium lauryl sulfate solution 3 times. After air-drying the filter, the filter was applied to an X-ray film (manufactured by Fuji Photo Film Co., Ltd., RX) at -80°C to obtain autoradiogram. As the result, a single and clear band was obtained respectively with both probes of AP-23 and AP-24, for the digestion products of Aspergillus oryzae chromosome with restriction enzymes.

4. Preparation of library

As the result of Southern hybridization described above, a band of about 6.5 kb was noted with the digestion product of Aspergillus oryzae-derived chromosomal DNA with Bgl II in the case of using AP-23 as the probe and a band of about 4.5 kb was noted with the digestion product with Hind III in the case of using AP-24. Bgl II and Hind III are restriction enzymes present in alkaline protease cDNA. It is thus considered that cloning of DNA fragments obtained with both restriction enzymes would cover cloning of alkaline protease gene over almost the entire region. Therefore, genomic library of Aspergillus oryzae using these restriction enzymes was prepared. Firstly, about 200 μg of the chro-

mosomal DNA of Aspergillus oryzae prepared in 1. above was digested at 37°C with 500 units each of Bgl II and Hind III (both manufactured by Takara Shuzo Co., Ltd.) overnight. After 0.6% agarose gel electrophoresis (at 30 V overnight). DNA fragment having a size of 5.0 to 7.0 kb (kilobase) and DNA fragment having a size of 3.0 to 5.0 kb were extracted and purified from the Bgl II digestion product and the Hind III digestion product, respectively, based on the size of a molecular weight marker simultaneously subjected to electrophoresis. Extraction and purification from agarose gel can be performed by the method of Maniatis et al. (supra). On the other hand, after digestion with BamH I which formed the same cohesive end as with Bgl II or digestion with Hind III using pUC19 as the vector, the terminus was dephosphorylated with alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd.) to prevent self ligation.

The DNA fragment of 5.0 to 7.0 kb as the Bgl II digestion product described above was mixed with pUC19 previously digested with BamH I, and the DNA fragment of 3.0 to 5.0 kb as the Hind III digestion product was likewise mixed with pUC19 previously digested with Hind III. Using "Ligation Kit"(manufactured by Takara Shuzo Co., Ltd.), ligation was performed. Competent cells (manufactured by Takara Shuzo Co., Ltd.) of Escherichia coli strain HB101 were transformed using the resulting mixture. A part of the transformed Escherichia coli was subjected to plating on L agar medium (1% Bacto-trypton, 0.5% yeast extract, 1% NaCl, 1.5% agar) supplemented with 50 µg/ml of ampicilin for determining frequency of transformation. The balance was supplemented with 50-fold volume of L-broth (1% Trypton, 0.5% yeast extract, 1% NaCl) supplemented with 50 µg/ml of ampicilin and cultured at 37°C overnight to amplify the colony. The colony was then stored at -80°C as a genomic library of Aspergillus oryzae.

5. Colony hybridization

Colony hybridization was performed as described below. Firstly, the genomic library of Aspergillus oryzae was applied to plating in about 10,000 clones per plate having a diameter of 150 mm, followed by incubation at 37°C overnight. As medium for the plate, L agar medium supplemented with 50 µg/ml of ampicillin was used. After the incubation, a nylon filter (manufactured by NEN, Colony/Plaque Screen) was gently placed on the agar to transfer the colony onto the nylon filter. The nylon filter onto which the colony had been transferred was lyzed by immersing the same in 0.5 N NaOH to denature DNA. The nylon filter was then immersed in 1 M Tris-hydrochloride (pH 7.5) solution to neutralize. After drying, the denatured DNA was fixed. The thus prepared filter was placed in a vinyl bag and hybridization was performed under the same conditions as in the Southern hybridization. As the result, several clones hybridizing with probes of AP-23 or AP-24 were obtained. From these clones, plasmid DNA was extracted by the alkaline-SDS method (Maniatis, supra) and its nucleotide sequence was determined by the dideoxy method. The nucleotide sequence coincided with the cDNA sequence of alkaline protease.

Plasmid carrying the Bgl II fragment of about 6.5 kb capable of hybridizing with the AP-23 probe and plasmid carrying the Hind III fragment of about 4.5 kb capable of hybridizing with the AP-24 probe were named pAP017 and pAP025, respectively. Using these plasmids, the following analysis was conducted.

6. Acquisition of genome gene of alkaline protease and determination of its DNA sequence

pAP017 and pAP025 obtained in procedure 5, above were digested with several restriction enzymes. Then, their fragment patterns were observed by agarose gel electrophoresis to prepare a restriction enzyme map. The results are shown in Fig. 1. The gene fractions contained in the two plasmids were overlapping between the Hind III site and the Bgl II site and almost the entire region of alkaline protease gene could be cloned by the plasmids. Thus, the DNA sequence was determined using appropriate restriction enzyme sites, as shown in Fig. 2. For determination of the DNA sequence, the dideoxy method was used. "M13 Sequencing Kit" (manufactured by Takara Shuzo Co., ltd.) was used for the reaction and "Apparatus for Electrophoresis for Determination of DNA Nucleotide Sequence" (manufactured by Takara Shuzo Co., Ltd.) was used for electrophoresis. Comparison of the nucleotide sequence of genomic gene with the nucleotide sequence of alkaline protease cDNA (Japanese Patent Application No. 63-170018) reveals that intron sequence at 3 sites in total was present in the alkaline protease gene, i.e., one at its prepro region and two at its mature protein region.

7. Determination of transcription initiation site

In order to analyze the promoter domain of the cloned alkaline protease gene, its transcription initiation site was determined. Known methods for determination of the transcription initiation site are the S1 mapping method and the primer extension method. In this run, the primer extension method was used. As the primer, oligonucleotide synthesized based on a reverse chain of DNA encoding 12 to 17 amino acids (within the preregion) of alkaline protease protein was used. The sequence of oligonucleotide is shown below.

AP-26: 5' CGC GGG AAG GAT AGC TCC 3'

The sequence of AP-26 was synthesized using "DNA Synthesizer (381A)" manufactured by Applied Biosystems Co., Ltd. The synthesized oligonucleotide was purified using "Oligonucleotide Purification Cartridge" manufactured by Applied Biosystems Co., Ltd. The terminal labeling of AP-26 was performed using [γ-^{32}P] ATP and T4 polynucleotide kinase. The reaction was carried out at 37°C for an hour in a solution of 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$ and 10 mM dithiothreitol (DTT). [γ-^{32}P] ATP which was not used for the labeling was removed using "NENSORB^{TM}20" column (manufactured by NEN). By the foregoing procedures, oligonucleotide having radioactivity of about 10^7 cpm per 1 µg DNA was obtained.

Next, about 3 µg of mRNA (Japanese Patent Application No. 63-170018) used as a template for cloning alkaline protease cDNA was mixed with about 10 ng of the AP-26 primer labeled with ^{32}P to make the volume 6 µl and, 1 µl of 10 x reverse transcription buffer (50 mM Tris-hydrochloride (pH 8.0), 500 mM KCl and 100 mM $MgCl_2$) was added thereto. After heat treatment at 70°C for 5 minutes, the mixture was allowed to stand at room temperature for 20 minutes to effect annealing of the primer and mRNA.

Next, 1 µl of 10 mM DTT, 20 mM dNTP (equimolar mixture of dATP, dGTP, dCTP and dTTP), 0.5 µl of ribonuclease inhibitor (117 units/µl, manufactured by Takara Shuzo Co., Ltd.) and 0.5 µl of reverse transcriptase (manufactured by Takara Shuzo Co., Ltd., 22 units/µl) were added to the solution. After reacting at 42°C for an hour, 10 µl of formamide solution (95% formamide, 0.1% xylene cyanol and 0.1% bromophenol blue) was added to the reaction mixture to terminate the reaction.

Then, 1 to 3 µl of the reaction solution obtained above was applied onto 6% acrylamide-urea gel. At the same time, the solution obtained by the dideoxy reaction using pAP017 as a template and AP-26 as a primer was applied to the same gel as a size marker. As a result, 3 bands were noted with the reaction subjected to the primer extension and 3 transcription initiation sites were present in the alkaline protease gene. The results are shown in Fig. 2. The sequence TATAAAT which is considered to be a so-called TATA box is present at the upstream by 30 to 40 nucleotides from the uppermost stream of the transcription initiation site thus determined. At a position upstream by 80 to 90 nucleotides, the sequence CCAAAT which is considered tobea CAAT box is present.

8. Ligation of alkaline protease genome gene

To ligate the alkaline protease genome genes cloned to the two DNA fragments (pAP017 and pAP025), the procedures shown in Fig. 5 were performed. Firstly, about 50 µg of pAP017 was digested with Nco I (manufactured by Takara Shuzo Co., Ltd.) and the terminus was rendered blunt, using "DNA Blunting Kit" (manufactured by Takara Shuzo Co., Ltd.). With the digested fragment was mixed 5 µg of BamH I linker (manufactured by Takara Shuzo Co., Ltd.). Using "DNA Ligation Kit" (manufactured by Takara Shuzo Co., Ltd.), both were ligated with each other. After purification by ethanol precipitation, DNA was digested with BamH I and Hind III (manufactured by Takara Shuzo Co., Ltd.). The DNA mixture was isolated by 1% agarose gel electrophoresis. The DNA fragment of about 1200 bp containing the 5'-site of alkaline protease genome gene was recovered and purified from the gel. Recovery of DNA from the gel can be performed according to the method of Maniatis et al. (supra). On the other hand, after digesting about 50 µg of pAP025 with Pst I (manufactured by Takara Shuzo Co., Ltd.), the terminus was rendered blunt, using "DNA Blunting Kit" (manufactured by Takara Shuzo Co., Ltd.). Likewise, 5 µg of BamH I linker was mixed with the digested fragment. Using "DNA Ligation Kit" (manufactured by Takara Shuzo Co., Ltd.), ligation was performed. After the ligation, purification was carried out by ethanol precipitation and DNA was digested with BamH I and Hind III.

The DNA digestion product was isolated by 1% agarose gel electrophoresis. The DNA fragment of about 1800 bp containing the 3'-site of alkaline protease genome gene was recovered and purified from the gel.

About 3 µg of the aforesaid 5'-site DNA fragment (about 1200 bp, BamH I/Hind III fragment) and about 3 µg of the 3'-site DNA fragment (about 1800 bp, BamH I/Hind III fragment) of alkaline protease were mixed with each other. After ligation by "DNA Ligation Kit", digestion was performed with BamH I. The digested DNA was isolated by 1% agarose gel electrophoresis and the DNA fragment having the desired size (about 3 kb) was recovered and purified. After the DNA fragment containing this alkaline protease genome gene was mixed with the BamH I-digested pUC19 to ligate them, the ligation product was transferred to competent cells of Escherichia coli strain JM109 (manufactured by Takara Shuzo Co., ltd.). A transformant which acquired ampicillin resistance was screened to obtain clone bearing the desired plasmid pAP1725. pAP1725 is DNA inserted with alkaline protease genome gene (about 3 kb) at the BamH I site of pUC19.

9. Preparation of expression vector

An expression vector using the promoter and terminator of alkaline protease gene was prepared by strategy shown in Fig. 6.

For isolating the terminator domain, 10 µg of pAP025 was digested with Afl II (manufactured by Takara Shuzo Co., Ltd.). Thereafter, the cleaved site was converted into the blunt end, using "DNA Blunting Kit" (manufactured by Takara

Shuzo Co., Ltd.). After purification by ethanol precipitation, further digestion was performed with Pst I (manufactured by Takara Shuzo Co., Ltd.). The digested fragments were isolated by 1% agarose gel electrophoresis. Among them, DNA fragment (about 500 bp) in the terminator domain of alkaline protease was extracted and purified from the agarose gel. On the other hand, after pUC19 which may be used as a vector was digested with Pst I and Hinc II (manufactured by Takara Shuzo Co., Ltd.) the cleaved site was dephosphorylated with alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd.). The cleaved vector (about 100 ng) was mixed with the terminator DNA fragment (about 500 bp) described above. After ligation of the mixture by "DNA Ligation Kit", Escherichia coli obtain JM109 (manufactured by Takara Shuzo Co., Ltd.) was transformed with the mixture. From the clone which acquired ampicillin resistance, plasmid DNA was extracted and screened to obtain the desired plasmid pAP044.

Next, for isolating the promoter domain, about 10 µg of pAP017 was digested with Nco I (manufactured by Takara Shuzo Co., Ltd.). Thereafter, the cleaved site was converted into the blunt end, using "DNA blunting Kit". After ethanol precipitation, DNA was mixed with 2 µg of Eco RI linker (manufactured by Takara Shuzo Co., Ltd.) followed by ligation using "DNA Ligation kit". The ligated DNA mixture was digested with Eco RI and Fsp I (manufactured by New England Biolab Co., Ltd.). The digested fragments were isolated by 1% agarose gel electrophoresis. Among the DNA fragments isolated, DNA fragment (about 1100 bp) containing the promoter domain of alkaline protease was extracted and purified from the agarose gel.

Then about 10 µg of plasmid pAP044 to which the terminator domain had been subcloned was digested with Eco RI and Sma I (manufactured by Takara Shuzo Co., Ltd.) followed by dephosphorylation by alkaline phosphatase. The deposphorylated product (about 100 ng) was mixed with the aforesaid promoter fragment (about 1100 bp, about 100 ng) to ligate them using "DNA Ligation Kit". Escherichia coli strain JM109 was transformed with the mixture. From the strain resistant to ampicillin, plasmid DNA was extracted and screened to obtain the desired plasmid pAP045. pAP045 is a plasmid ligated with the promoter and terminator of alkaline protease and is a vector capable of expression by inserting a heterologous gene into the BamH I site which is the unique recognition site.

Claims

1. A plasmid comprising a promoter sequence derived from the *Aspergillus oryzae* alkaline protease genome gene, which sequence is as shown in Figure 2, or a functional equivalent thereof.

2. A plasmid comprising a terminator sequence derived from the *Aspergillus oryzae* alkaline protease genome gene, which sequence is as shown in Figure 3, or a functional equivalent thereof.

3. A plasmid according to claim 1 or claim 2 which is an expression vector.

4. A gene expression unit comprising a promoter sequence derived from the *Aspergillus oryzae* alkaline protease genome gene, which sequence is as shown in Figure 2, or a functional equivalent thereof, and a terminator sequence derived from the *Aspergillus oryzae* alkaline protease genome gene, which sequence is as shown in Figure 3, or a functional equivalent thereof.

5. An expression vector comprising a gene expression unit according to claim 4.

6. A plasmid comprising a genome gene of alkaline protease derived from *Aspergillus oryzae*, the gene having the restriction enzyme map shown in Figure 1.

7. A plasmid comprising a genome gene of alkaline protease derived from *Aspergillus oryzae* and having the nucleotide sequence shown in Figure 4.

Patentansprüche

1. Plasmid, umfassend eine Promotorsequenz, die von dem Gen der alkalischen Protease aus dem Genom von Aspergillus oryzae stammt, wobei die Sequenz in Figur 2 gezeigt ist, oder ein funktionelles Äquivalent davon.

2. Plasmid, umfassend eine Terminatorsequenz, die von dem Gen der alkalischen Protease aus dem Genom von Aspergillus oryzae stammt, wobei die Sequenz in Figur 3 gezeigt ist, oder ein funktionelles Äquivalent davon.

3. Plasmid nach Anspruch 1 oder 2, das ein Expressionsvektor ist.

4. Genexpressionseinheit, umfassend eine Promotorsequenz, die von dem Gen der alkalischen Protease aus dem Genom von Aspergillus oryzae stammt, wobei die Sequenz in Figur 2 gezeigt ist, oder ein funktionelles Äquivalent davon, und eine Terminatorsequenz, die von dem Gen der alkalischen Protease aus dem Genom von Aspergillus oryzae stammt, wobei die Sequenz in Figur 3 gezeigt ist, oder ein funktionelles Äquivalent davon.

5. Expressionsvektor, umfassend eine Genexpressionseinheit nach Anspruch 4.

6. Plasmid, umfassend ein aus dem Genom von Aspergillus oryzae stammendes Gen der alkalischen Protease, das die in Figur 1 gezeigte Restriktionsenzymkarte hat.

7. Plasmid, umfassend ein aus dem Genom von Aspergillus oryzae stammendes Gen der alkalischen Protease, das die in Figur 4 gezeigte Nucleotidsequenz hat.

Revendications

1. Plasmide comprenant une séquence de promoteur obtenue à partir d'un gène du génome de la protéase alcaline d'Aspergillus oryzae, laquelle séquence est telle que représentée dans la figure 2, ou un de ses équivalents fonctionnels.

2. Plasmide comprenant une séquence de terminateur obtenue à partir d'un gène du génome de la protéase alcaline d'Aspergillus oryzae, laquelle séquence est telle que représentée dans la figure 3, ou un de ses équivalents fonctionnels.

3. Plasmide selon la revendication 1 ou la revendication 2, qui est un vecteur d'expression.

4. Unité d'expression d'un gène comprenant une séquence de promoteur obtenue à partir d'un gène du génome de la protéase alcaline d'Aspergillus oryzae, laquelle séquence est telle que représentée dans la figure 2, ou un de ses équivalents fonctionnels, et une séquence de terminateur obtenue à partir d'un gène du génome de la protéase alcaline d'Aspergillus oryzae, laquelle séquence est telle que représentée dans la figure 3, ou un de ses équivalents fonctionnels.

5. Vecteur d'expression comprenant une unité d'expression d'un gène conforme à la revendication 4.

6. Plasmide comprenant un gène du génome de la protéase alcaline obtenu à partir d'Aspergillus oryzae, le gène ayant la carte d'enzyme de restriction représentée dans la figure 1.

7. Plasmide comprenant un gène du génome de la protéase alcaline obtenu à partir d'Aspergillus oryzae et ayant la séquence de nucléotides représentée dans la figure 4.

FIG. 1

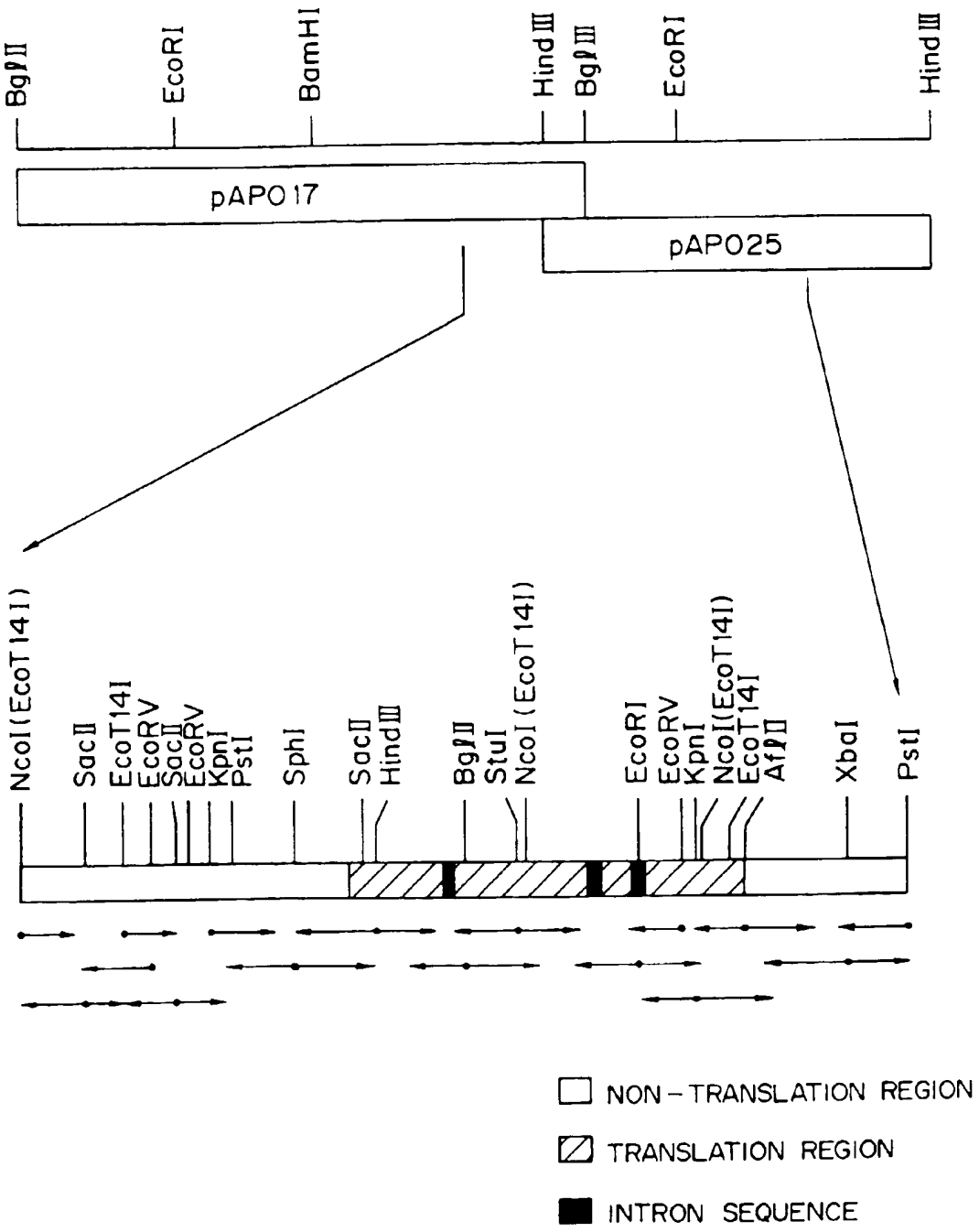

BglII
EcoRI
BamHI
HindIII
BglII
EcoRI
HindIII
pAPO17
pAPO25
NcoI(EcoT141)
SacII
EcoT141
EcoRV
SacII
EcoRV
KpnI
PstI
SphI
SacII
HindIII
BglII
StuI
NcoI(EcoT141)
EcoRI
EcoRV
KpnI
NcoI(EcoT141)
EcoT141
AflII
XbaI
PstI
NON-TRANSLATION REGION
TRANSLATION REGION
INTRON SEQUENCE

FIG. 2

```
                                                                      80
CCATGGTTAT TCTGCGGAAG CGAAACCACC CTCCCACCCA AACAGGGCTA ATGTGCCCAG GTCCTGATAC CATCAGAAGA
                                                                     120
CCTCCAGGAG CACATGCCTG TTCGCATAAC CGTGGTGTAG CACCAGGAAT TGCTTAGCTT AGCTTCTTCG ACTGGGGGGC
                                                                     240
CAGAAAGTGC TTATCGCAAA GATCCCACTT CTTTGTGTGA TAGCCCCTCC CGCGGCCCTT GATCAAGCCG TTCTCGCTCG
                                                                     320
CCCATACCGA AACCGCGATA TTATAGGTGC ACATGGTTAT TATTCTTTTT CTTTTTCTTT TTCTTTGCTT CTCATGCAGC
                                                                     400
CCCATACGTT GCCGAATTTG GCTACACCTT GGGGCTCATT CTTCGAAGTT TAGATTCCGA CAAGACCTCA GCACCCAATC
                                                                     480
AAAACCCTTG ATTCCTGATA AAAGACGTGG AAAAAAGCGG ATATCGCGTG AGGATGCCAA GCAAAGGGAA TGGGTCACAT
                                                                     560
TGATCTCTGT CGCGCTGTTA GGATGATCTT CACTCCTAAA GGCATCGCCC GCGGCATTAG GCCCTTCCTG TCCAAGATAT
                                                                     640
CGGTTACTCC TCTCATTATG GCGAGCTACT TTGTGAATTA ATTGACTGAG GGATATACCA CCTTCCCTTT GAAGGTACCG
                                                                     720
AGCCACTACC TTGAGCGTTA GTTACTTTTT CGAGGAAAGC ATCCTATGCT AGTCTCTGCC AATCACTGCA GCGTCGACAA
                                                                     800
CTTGCCATAG CCTTGTGTTC TTCACGGTCT ATCGGAACAC CCGTTCATGA CTGAAAGGGG TCAGCGTCCG TGGTGGTCAA
                                                                     880
CATCATTCTC ATCTTTCATC ATGCCCGCTG ATTGATAGAG TAATTTCCGG TGGAGCACAA CGCCGTCCTC TGACATGCAA
                                                                     960
TGTCACCCTG TAAGTTTCAA CTACAATCTG TAGTACAGAG CATCCTTGTC ATTGCATGCT GTGCAAGTGA TCCAAATCCG
                                                                    1040
TAGAACTTGC TCGAGAACAG GGAAATATAG AACTCCTGAA GGTTATAAAT ACCACATGCA TCCCTCGTCC ATCCTCACTT
 ↓  ↓  ↓
CCATCATCAA GCCAGCGGTT TCTATCCTCC GACTTGAGTT GTTCTTGCGC ATCTTTACAA TCTTCTCATC
```

——: CAAT BOX
═══: TATA BOX
↓ : TRANSCRIPTION INITIATION SITE

FIG. 3

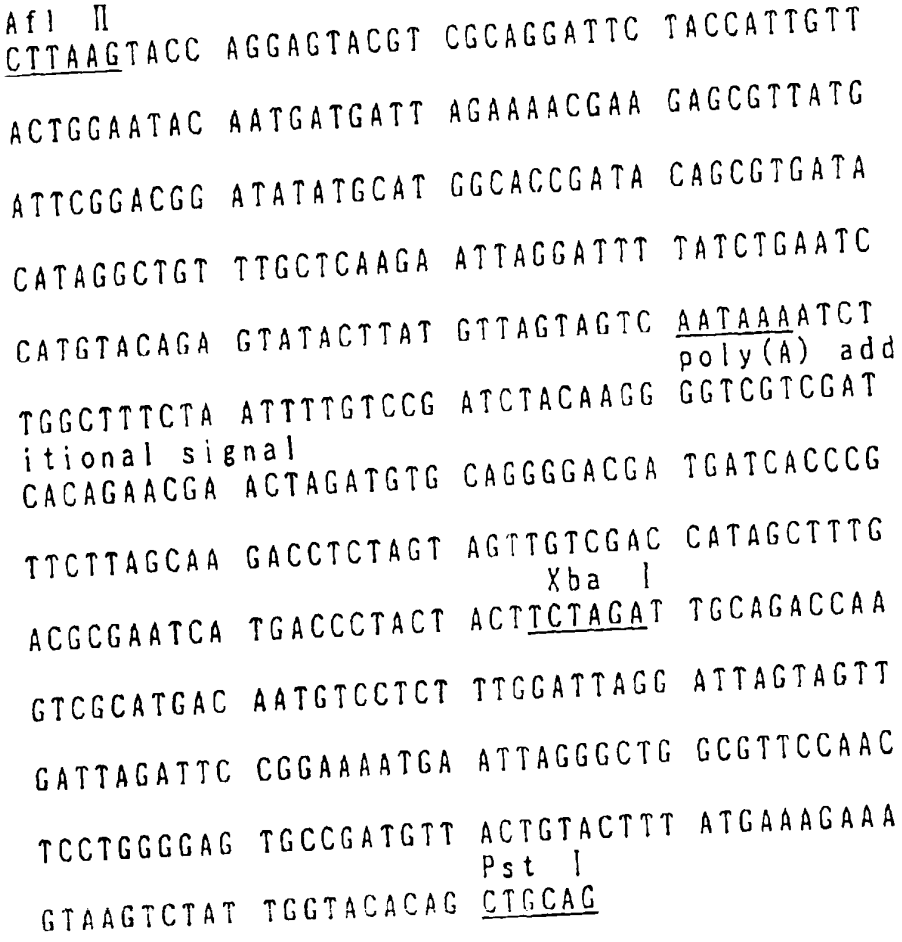

Afl II
CTTAAGTACC AGGAGTACGT CGCAGGATTC TACCATTGTT
ACTGGAATAC AATGATGATT AGAAAACGAA GAGCGTTATG
ATTCGGACGG ATATATGCAT GGCACCGATA CAGCGTGATA
CATAGGCTGT TTGCTCAAGA ATTAGGATTT TATCTGAATC
CATGTACAGA GTATACTTAT GTTAGTAGTC AATAAAATCT
poly(A) add
TGGCTTTCTA ATTTTGTCCG ATCTACAAGG GGTCGTCGAT
itional signal
CACAGAACGA ACTAGATGTG CAGGGGACGA TGATCACCCG
TTCTTAGCAA GACCTCTAGT AGTTGTCGAC CATAGCTTTG
Xba I
ACGCGAATCA TGACCCTACT ACTTCTAGAT TGCAGACCAA
GTCGCATGAC AATGTCCTCT TTGGATTAGG ATTAGTAGTT
GATTAGATTC CGGAAAATGA ATTAGGGCTG GCGTTCCAAC
TCCTGGGGAG TGCCGATGTT ACTGTACTTT ATGAAAGAAA
Pst I
GTAAGTCTAT TGGTACACAG CTGCAG

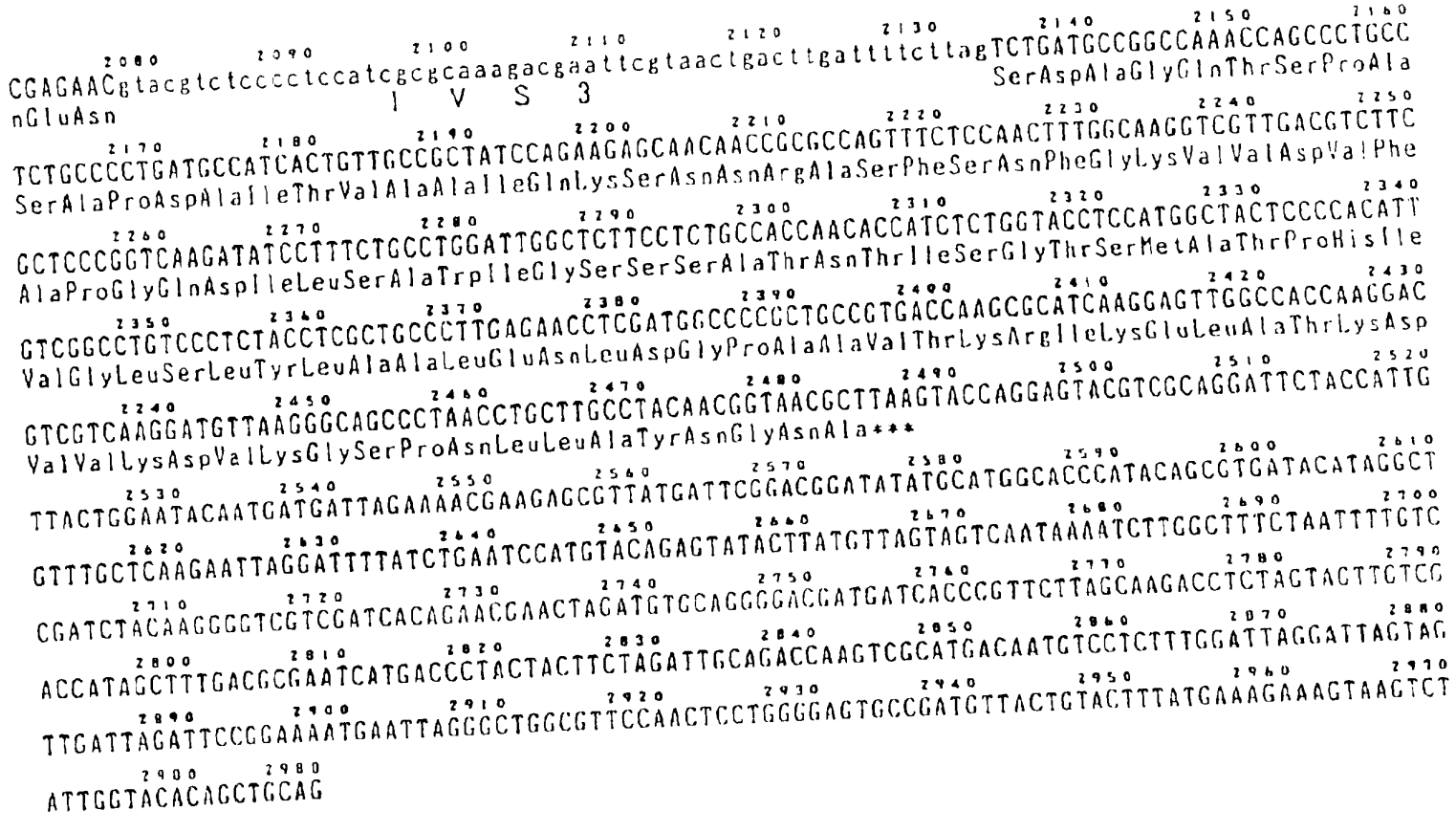
FIG. 4A


MetGlnSerIleLysArgThrLeuLeuLeuLeuGlyAlaIleLeuProAlaValLeuGly
AlaProValGlnGluThrArgArgAlaAlaGluLysLeuProGlyLysTyrIleValThrPheLysProGlyIleAspGluAlaLysIle

FIG. 4B


1270 1280 1290 1300 1310 1320 1330 1340 1350
CAGGAGCATACCACCTGGGCTACCAACATTCACCAGCGCAGTCTGGAGCGTCGTGGCGCCACTGGCGGTGATCTTCCTGTCGGTATTGAG
GlnGluHisThrThrTrpAlaThrAsnIleHisGlnArgSerLeuGluArgArgGlyAlaThrGlyGlyAspLeuProValGlyIleGlu
1360 1370 1380 1390 1400 1410 1420 1430 1440
CGCAACTACAAGATCAACAAGTTCGCCGCCTATGCAGGCTCTTTCGACGATGCTACCATTGAGGAGATTCGCAAGAACGAAGATgtttgt
ArgAsnTyrLysIleAsnLysPheAlaAlaTyrAlaGlySerPheAspAspAlaThrIleGluGluIleArgLysAsnGluAsp
1450 1460 1470 1480 1490 1500 1510 1520 1530
ggtcatccgctcgcatttttgaatgacagctaactcgcgcccagGTTGCCTACGTCGAGGAGGACCAGATCTACTACCTCGATGGCCTGA
ValAlaTyrValGluGluAspGlnIleTyrTyrLeuAspGlyLeuT
I V S 1
1540 1550 1560 1570 1580 1590 1600 1610 1620
CTACCCAGAAGAGTGCCCCCTGGGGTCTGGGCAGCATTTCCCACAAGGGCCAGCAGAGCACCGACTACATCTACGACACTAGTGCCGGCG
hrThrGlnLysSerAlaProTrpGlyLeuGlySerIleSerHisLysGlyGlnGlnSerThrAspTyrIleTyrAspThrSerAlaGlyG
1630 1640 1650 1660 1670 1680 1690 1700 1710
AGGGCACCTATGCCTACGTGGTGGATAGCGGTGTCAATGTCGACCATGAGGAGTTCGAGGGCCGCGCCAGCAAGGCCTACAACGCTGCCG
luGlyThrTyrAlaTyrValValAspSerGlyValAsnValAspHisGluGluPheGluGlyArgAlaSerLysAlaTyrAsnAlaAlaG
1720 1730 1740 1750 1760 1770 1780 1790 1800
GTGGTCAGCATGTGGACAGCATTGGCCATGGCACCCACGTTTCCGGCACCATTGCTGGCAAGACTTATGGTATCGCCAAGAAGGCCAGCA
lyGlyGlnHisValAspSerIleGlyHisGlyThrHisValSerGlyThrIleAlaGlyLysThrTyrGlyIleAlaLysLysAlaSerI
1810 1820 1830 1840 1850 1860 1870 1880 1890
TCCTTTCGGTCAAAGTTTTCCAGGGTGAATCGAGCAGCACTTCCGTCATTCTTGACGGCTTCAACTGGGCTGCCAACGACATTGTTAGCA
leLeuSerValLysValPheGlnGlyGluSerSerSerThrSerValIleLeuAspGlyPheAsnTrpAlaAlaAsnAspIleValSerL
1900 1910 1920 1930 1940 1950 1960 1970 1980
AGAAGCGTACCAGCAAGGCTGCAATCAACATGAGCTTGGgtgagtttacattgttcttctctacttggaacgcgcgagcgctaatttcaa
ysLysArgThrSerLysAlaAlaIleAsnMetSerLeuG
I V S 2
1990 2000 2010 2020 2030 2040 2050 2060 2070
aaacacagGCGGTGGCTACTCTAAGGCTTTCAACGATGCGGTCGAGAACGCATTCGAGCAGGGTGTTCTCTCGGTTGTCGCTGCCGGTAA
lyGlyGlyTyrSerLysAlaPheAsnAspAlaValGluAsnAlaPheGluGlnGlyValLeuSerValValAlaAlaGlyAs

FIG. 4C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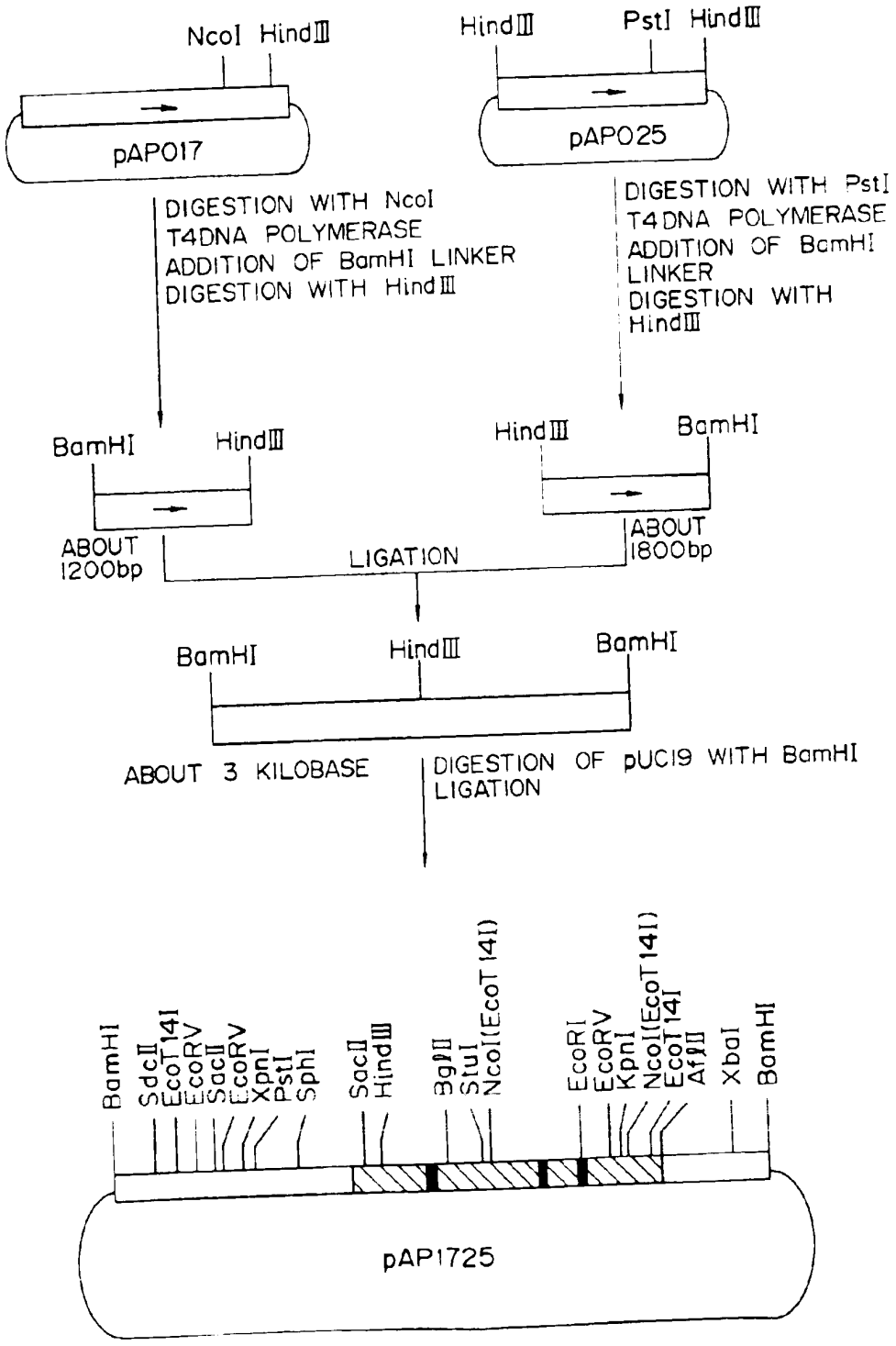
SerAspAlaGlyGlnThrSerProAla
nGluAsn
I V S 3
2170 2180 2190 2200 2210 2220 2230 2240 2250
TCTGCCCCTGATGCCATCACTGTTGCCGCTATCCAGAAGAGCAACAACCGCGCCAGTTTCTCCAACTTTGGCAAGGTCGTTGACGTCTTC
SerAlaProAspAlaIleThrValAlaAlaIleGlnLysSerAsnAsnArgAlaSerPheSerAsnPheGlyLysValValAspValPhe
2260 2270 2280 2290 2300 2310 2320 2330 2340
GCTCCCGGTCAAGATATCCTTTCTGCCTGGATTGGCTCTTCCTCTGCCACCAACACCATCTCTGGTACCTCCATGGCTACTCCCCACATT
AlaProGlyGlnAspIleLeuSerAlaTrpIleGlySerSerSerAlaThrAsnThrIleSerGlyThrSerMetAlaThrProHisIle
2350 2360 2370 2380 2390 2400 2410 2420 2430
GTCGGCCTGTCCCTCTACCTCGCTGCCCTTGAGAACCTCGATGGCCCCGCTGCCGTGACCAAGCGCATCAAGGAGTTGGCCACCAAGGAC
ValGlyLeuSerLeuTyrLeuAlaAlaLeuGluAsnLeuAspGlyProAlaAlaValThrLysArgIleLysGluLeuAlaThrLysAsp
2240 2450 2460 2470 2480 2490 2500 2510 2520
GTCGTCAAGGATGTTAAGGGCAGCCCTAACCTGCTTGCCTACAACGGTAACGCTTAAGTACCAGGAGTACGTCGCAGGATTCTACCATTG
ValValLysAspValLysGlySerProAsnLeuLeuAlaTyrAsnGlyAsnAla***
2530 2540 2550 2560 2570 2580 2590 2600 2610
TTACTGGAATACAATGATGATTAGAAAACGAAGAGCGTTATGATTCGGACGGATATATGCATGGCACCCATACAGCGTGATACATAGGCT
2620 2630 2640 2650 2660 2670 2680 2690 2700
GTTTGCTCAAGAATTAGGATTTTATCTGAATCCATGTACAGAGTATACTTATGTTAGTAGTCAATAAAATCTTGGCTTTCTAATTTTGTC
2710 2720 2730 2740 2750 2760 2770 2780 2790
CGATCTACAAGGGGTCGTCGATCACAGAACGAACTAGATGTGCAGGGGACGATGATCACCCGTTCTTAGCAAGACCTCTAGTAGTTGTCG
2800 2810 2820 2830 2840 2850 2860 2870 2880
ACCATAGCTTTGACGCGAATCATGACCCTACTACTTCTAGATTGCAGACCAAGTCGCATGACAATGTCCTCTTTGGATTAGGATTAGTAG
2890 2900 2910 2920 2930 2940 2950 2960 2970
TTGATTAGATTCCCGAAAATGAATTAGGGCTGGCGTTCCAACTCCTGGGGAGTGCCGATGTTACTGTACTTTATGAAAGAAAGTAAGTCT
2900 2980
ATTGGTACACAGCTGCAG

FIG. 5


NcoI HindIII
pAP017
HindIII PstI HindIII
pAP025
DIGESTION WITH NcoI
T4DNA POLYMERASE
ADDITION OF BamHI LINKER
DIGESTION WITH HindIII
DIGESTION WITH PstI
T4DNA POLYMERASE
ADDITION OF BamHI
LINKER
DIGESTION WITH
HindIII
BamHI HindIII
ABOUT
1200bp
HindIII BamHI
ABOUT
1800bp
LIGATION
BamHI HindIII BamHI
ABOUT 3 KILOBASE
DIGESTION OF pUC19 WITH BamHI
LIGATION
BamHI
SdcII
EcoT141
EcoRV
SacII
EcoRV
XpnI
PstI
SphI
SacI
HindIII
BglII
StuI
NcoI(EcoT141)
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EcoRV
KpnI
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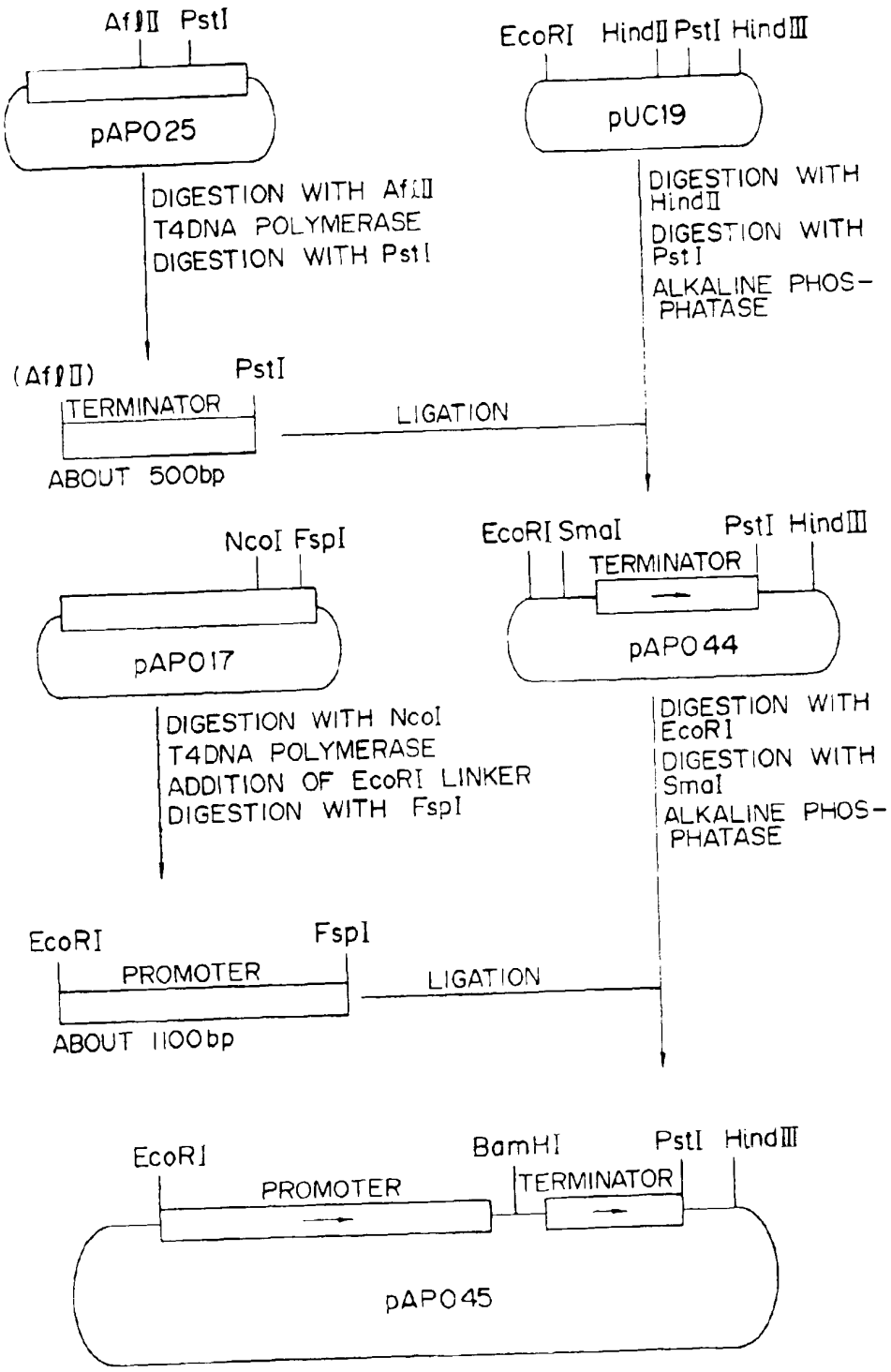
EcoT141
AflII
XbaI
BamHI
pAP1725

FIG. 6


AfIII PstI
pAP025
DIGESTION WITH AfIII
T4DNA POLYMERASE
DIGESTION WITH PstI
EcoRI HindII PstI HindIII
pUC19
DIGESTION WITH HindII
DIGESTION WITH PstI
ALKALINE PHOSPHATASE
(AfIII)
PstI
TERMINATOR
ABOUT 500bp
LIGATION
NcoI FspI
pAP017
DIGESTION WITH NcoI
T4DNA POLYMERASE
ADDITION OF EcoRI LINKER
DIGESTION WITH FspI
EcoRI SmaI
PstI HindIII
TERMINATOR
pAP044
DIGESTION WITH EcoRI
DIGESTION WITH SmaI
ALKALINE PHOSPHATASE
EcoRI
FspI
PROMOTER
ABOUT 1100bp
LIGATION
EcoRI
BamHI
PstI HindIII
PROMOTER
TERMINATOR
pAP045